INVESTIGATIONS INTO THE OUTER SURFACE OF PATHOGENIC TREPONEMA PALLIDUM

Onderzoekingen naar het oppervlak van pathogene *Treponema pallidum*

PROEFSCHRIFT

ter verkrijging van de graad van doctor
aan de Erasmus Universiteit Rotterdam
op gezag van de Rector Magnificus
Prof. Dr. A.H.G. Rinnooy Kan
en volgens besluit van het college van dekanen.

De openbare verdediging zal plaats vinden op
vrijdag 4 december 1987 om 15.45 uur

door

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geboren te Assen

krips repro meppel

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In dit proefschrift zijn de volgende wetenschappelijke publicaties opgenomen:

J.J. van der Sluis, E.C. van Reede and M. Boer. Immunoglobulin G subclasses of fluorescent anti-Treponema pallidum antibodies: Evidence for sequential development of specific anti-T. pallidum immunoglobulin G responses in patients with early syphilis. J. Clin. Microbiol. 24, 418-423 (1986)

J.J. van der Sluis, F.J.W. ten Kate, V.D. Vuzevski and E. Stolz.
Light- and electron microscopic study on Treponema pallidum (Nichols strain) infected rabbit testes, with special reference to the nature of deposited mucopolysaccharides and location of treponemes. Accepted by Genitourinary Medicine, 1986.

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The inaccesability of anti-treponemal antibodies to the outer membrane of adherent Treponema pallidum (Nichols strain). Submitted for publication.

In de periode van het bewerken verschenen de volgende, niet in het proefschrift opgenomen wetenschappelijke publicaties:

J.J. van der Sluis and M. Boer.
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Abbreviations used:

Serologic reactions for syphilis:

VDRL = Venereal Disease Research Laboratory test

FTA-ABS = Fluorescent Treponemal Antibody-Absorption test

TPI = Treponema pallidum Immobilisation test

TPHA = Treponema pallidum Haemagglutination test

Mitogens:

Con A = Concanavalin A

PHA = Phytohaemagglutinin

PWM = Pokeweed mitogen

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CHAPTER 1.

TREPONEMATOSES AND THEIR CAUSATIVE AGENTS.

1.1. Introduction

The treponematoses occurring in humans consist of four distinct, clinically dissimilar, infectious diseases caused by three species of treponemes. Treponema pallidum is the etiological agent of venereal syphilis as well as the non-venereal endemic syphilis. Treponema pertenue is responsible for yaws and Treponema carateum causes pinta. The three latter diseases together are referred to as the endemic treponematoses (1).

Until recently none of the pathogenic treponemes had been cultured in vitro. However, T. pallidum and T. pertenue have been routinely passaged in rabbits. Upon isolation, these spirochetes ressemble each other so closely that they cannot be distinguished from each other either morphologically or serologically (2). This is also supported by biochemical studies. Miao and Fieldsteel (3) concluded from DNA-reassociation studies that T. pallidum and T. pertenue appeared to be identical within the limits of the resolution of their technique. Thornburg and Baseman (4) demonstrated almost identical blotting patterns of one and two dimensional polyacrylamide gelelectrophoresis sepatated polypeptides from both types of treponemes.

A common characteristic of the pathogenic treponemes is that they cause a chronic disease, which progresses in clinically distinct stages. Early and late stages are usually easily recognized. The complications of the late stages can be serious and can result in malformations (1).

Until the start of mass campaigns by the World Health Organisation (about 1950), the endemic treponematoses were among mankinds most commom afflictions. They were confined to the warm, either arid or humid rural areas within the Tropics. For instance, it was estimated that about 50 % of the 400 million people living in these areas were exposed to infection with T. pertenue. As a result of the mass campaigns, the incidence of these diseases has largely diminished. However, in a recent investigation thousands of new patients were found in Mali, Mauretania, Niger and Upper Volta. This suggests an incidence of these diseases in these regions approaching the pre-campaign level. Africa still remains the most important reservoir of the endemic treponematoses. In view of these facts, the risk of a new spread of the disease still exists (1). Moreover, in a recent report on the incidence of yaws in Surinam between 1976 and 1981, (5) it appeared that in certain districts, a high incidence

of only serologically apparent treponemal infection existed in school children. In contrast to the classical multiple frambesiform lesions, the majority of these children showed very mild symptoms, which resembled secondary syphilis. This attenuated form of the disease increases the risk of the diagnosis of yaws being overlooked.

Syphilis occurs throughout the world as sporadic cases, which concentrate in urban areas. According to the World Health Organization there has been a world wide increase of primary and secondary syphilis from the low levels achieved after World War II. In the western world the incidence of the disease is variable. The high incidence during and after World War II fell to a low level in the 1950s (WHO reports 1975 and 1977 cited in reference 6). Reliable figures on the incidence in the post-War era are available from the United States and the United Kingdom. The incidence in the United States decreased from 75.6 per 100.000 inhabitants in 1947 to 4.1 in 1956. This figure gradually increased to 11.9 in 1976. Thereafter, it fell slightly. In England and Wales an analogous pattern in incidence was noted (6). In The Netherlands a similar trend seems to exist. Until 1976, when registration of cases of infectious syphilis became compulsory by law, no accurate registration existed. The number of patients with primary and secondary syphilis before 1976 were obtained by retrospective inquiries from the general practitioners and showed the lowest level in 1959. From this year on, there was a gradual increase. The number of registered cases of these two stages of syphilis increased from 691 in 1976 to 1169 in 1980. Since then this number has stabilized at around 1000 (7).

Two trends have been implicated in the world wide increase of syphilis: 1. The high proportions of infections in the male homosexuals, especially in the western world. 2. The reduction in the protection against syphilis by the elimination of endemic treponematoses, in areas where the mass campaigns have been highly successful (6).

A feature of syphilis not occurring in endemic treponematoses is the transplacental infection of the foetus, causing congenital syphilis.

1.2. Taxonomy.

The agents causing the treponematoses belong to the order Spirochaetales, family of the Treponemataceae, genus Treponema. The Spirochaetales comprise the group of flexuous, thin, gram-negative, chemoheterotrophic, helical-shaped micro-organisms which differ morphologically from the other prokaryotes by the

presence of an axial fibril, known also as an endoflagellum, axistyle, or axial filament (8).

1.3. Structure of treponemes.

The initial identification and morphological description of treponemes in primary syphilitic lesions in 1905 (9) was accompanied in the same year by the report on the presence of treponemes in the ulcers of two patients suffering from yaws (10). The agent causing pinta, T. carateum, was first described in 1938 (11). Due to their small transverse dimensions, the treponemes cannot be observed using an ordinary light microscope. Using darkfield microscopy, they appear as thin silver threads coiled like a corkscrew, moving with a characteristic rapid spinning motion. In 1928 Noguchi (12) gave a detailed, exact description of the morphology of treponemes from his light-microscopic

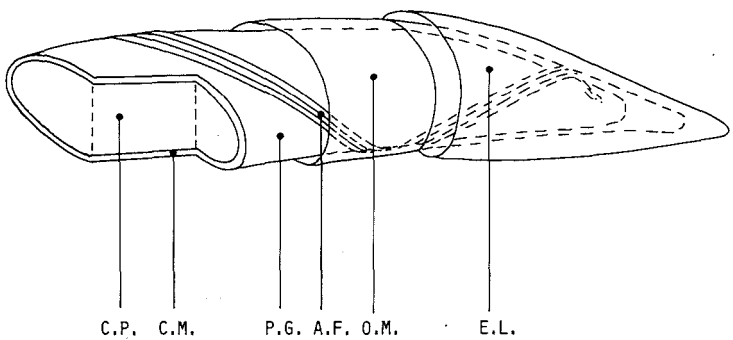

Fig. 1. Schematic representation of the structure of a pathogenic treponeme. C.P.= cytoplasm; C.M.= cytoplasmic membrane; P.G.= peptidoglycan layer; A.F.= axial filaments; O.M.= outer membrane; E.L.= extracellular layer.

observations. T. pallidum and T. pertenue were morphologically completely identical. Both microbes had a length of 8-11 µm and a width of 0.25 to 0.30 µm. They had tapered ends and possessed regular windings which were arranged in a fixed pattern with a wavelength of 1 µm. The cell division occurred by binary fission. Noguchi predicted the presence of spring-like axial filaments

and a contractile cytoplasm. Moreover, he expected the axial filaments to have much in common with the flagellae of bacteria. This was based on their morphology, the staining characteristics and a motility function.

Many of these details have been confirmed by electron microscopic observations. Swain (13) did not find real exogenic flagellae, but observed bundles of fibrils which were twisted spirally around the treponemes and were present along the whole length of the microbes. Different types of treponemes possessed a different number of fibrils. In T.pallidum mostly three, sometimes four fibrils were present. The most important structures which could be observed were the cytoplasmic body, the cell-wall and the fibrils which were located inside this cell-wall. Improvements in the technique and the staining procedures allowed more detailed observations without affecting the above mentioned essentials (reviewed in 14). A schematic representation of the structure of the treponemes is shown in Fig 1.

1.3.1 Extra-cellular layer.

In 1963 Christiansen postulated the presence of a mucous layer covering the treponemal outer membrane. This protective cover may explain the presence of pathogenic treponemes together with anti-treponemal antibodies of high titre in a host (15). The time lapse in antigen-antibody reactions in those serological reactions, which use live treponemes as an antigen, has been ascribed to the presence of a protective cover around the treponemes. It has been suggested that before seroreactivity can be demonstrated, this layer has to be altered or destroyed (16,17,18). Electron microscopic studies on sections of testes obtained from infected rabbits and which had been stained with ruthenium red, revealed dense deposits of acid mucopolysaccharides or closely related substances in areas rich in treponemes (19,20). However, after the isolation of the treponemes a large part of this material had disappeared from the surface of the treponemes (19). The conclusion of these authors that treponemes are covered with a layer of mucopolysaccharides has been refuted by Hovind-Hougen (14), because of the difficulty to observe structural details of the treponemes in the electron micrographs of Zeigler et al (19) and the symmetrical appearance of the outer membrane of the treponemes in the pictures of Fitzgerald et al (20). Accordingly, it was stated that electron microscopy was as yet unable to provide convincing evidence for the presence of a protective layer on cells of T. pallidum.

Alderette and Baseman (21) demonstrated a loose and avid binding of host serum proteins on the surface of pathogenic T.pallidum, Nichols strain. These

loosely bound proteins could be removed by washing. However, several avidly bound proteins remained on the treponemal surface. These proteins included a_2-macroglobulin, transferrin, ceruloplasmin, albumin, immunoglobulins G and M and the third complement component. These avidly bound proteins could only be removed by protease digestion with the concurrent release of treponemal proteins. Furthermore, fibronectin (54,59,60) and host-derived class I Major histocompatability Complex antigens (22) have been shown to be associated with the treponemal surface. Fibronectin has been implicated as the cellular ligand in the adherence of treponemes to cultured cells (54).

Agglutination of T. pallidum by wheat germ agglutinin and soy-bean agglutinin has been interpreted as evidence for the presence of hyaluronic acid and chondroitin sulphate as consituents of the acid mucopolysaccharides on the treponemal surface (20). However, the specificity of these agglutinins for N-acetyl-D-glucosamin and N-acetyl-D-galactosamin respectively may also point to reactivity to treponemal outer membrane glycoproteins or with associated host proteins.

1.4 Treponemes in vitro.

Until recently, in vitro culture of treponemes was not possible and still it is not possible to cultivate a large number of treponemes needed for biochemical analysis. Since the only reliable method to cultivate a large number of T. pallidum and T. pertenue is by infecting experimental animals, mostly rabbits, there is a problem concerning the purity of the harvested treponemes. Methods used to separate treponemes from host tissue include gradient centrifugation techniques (23,24,25) and continuous particle electrophoresis (26). The isolation using these techniques result in non-viable treponemes accompanied by the risk of loss or modification of treponemal constituents. On the other hand, unwashed treponemes carry absorbed host proteins on their surface (21). However, recently it has been shown that density gradient centrifugation on Percoll gradients yielded suspensions of viable treponemes that were free of host proteins (27,60).

Attempts have been made to define the optimal conditions essential for the successful cultivation of virulent treponemes. They include studies aimed at the development of experimental methods that prolong the motility and virulence of treponemes in artificial media and studies directed at the elucidation of the metabolic requirements of treponemes. Most of this research has centered on T. pallidum. Studies on the metabolic capacities of the treponemes have been hampered by the fact that in vitro culturing of

treponemes is not yet possible. First, this means that all studies were performed under non-permissive growth conditions and that full biosynthetic capacities of the treponemes may not have been utilized. Second, the isolation of the treponemes from the testes which results in contamination with eukaryotic cells or enzymes must be considered and rigorous controls are thus neccessary. Third, the treponemes are isolated from the testicular tissue at the moment of active growth. The catabolic and anabolic capacities of the treponemes could be the completion of processes already started in vivo and/or constitute the adaptation to in vitro conditions. Since in vitro multiplication of treponemes in cell-free systems is not possible as yet, it could impose severe restrictions on biochemical pathway studies and the interpretation of results.

1.4.1 Treponemes in suspension.

T. pallidum has been considered to be an anaerobic microbe (29). Anaerobic incubation indeed prolongs its motility in vitro. However, a sustained motility is only possible for a limited period of time and eventually the microbes die. The anaerobic requirement of the pathogenic treponemes is further challenged by their growth in vivo in highly vascularized tissues.

It has been known for a long time that the survival of treponemes is prolonged in media containing compounds rich in sulfhydryl (SH-) groups. Thioglycolate (29) and a combination of glutathion and cysteine (30) have been used. More recently, dithiothreitol has been added to this list (31). Two roles have been ascribed to these compounds (32,33,34). First, they contribute in maintaining a low redox potential, thereby minimizing oxygen toxicity and second, as reduced form they are required by the organisms for active metabolism (33).

Cox and Barber (35) showed that virulent T. pallidum consumed oxygen at a rate similar to that of the known aerobic spirochaete, Leptospira B_{16}. The amount of oxygen taken up was dependent on the number of treponemes. It was cyanide sensitive, indicating the presence of a cytochrome oxidase. Inhibition of oxygen uptake by azide, chlorpromazide and amital further suggested a functional electron transport system for the oxydation of reduced NADH. Lysko and Cox (36) presented evidence that energy was derived from terminal electron transport coupled to oxidative phosphorylation.

Studies on the catabolism of T. pallidum showed that from a total of 22 different carbon sources only glucose and pyruvate were used. These substances were catabolised to CO_2 and acetate as end products. (37,38). The accumulation

of acetate has been interpreted as evidence for the absence of a functional Kreb's cycle, although the enzymes isocitrate dehydrogenase and malate dehydrogenase have been demonstrated (38). It has been shown that the amount of glucose that was degraded correlated with the amount of oxygen that was present (39,40). The manner in which CO_2 was released from glucose and pyruvate was compatible with functional von Embden-Meyerhof-Parnas and hexose monophosphate pathways (38). This was supported by the demonstration of the major enzyme functions of both pathways in cell-free lysates of T. pallidum (38). From experiments in which treponemes were incubated at different dissolved oxygen concentrations (DOC), it appeared that the treponemes that were kept at high DOC consumed less oxygen and produced less acetate than treponemes kept at intermediate DOC. At high DOC pyruvate accumulated. This suggested that at high DOC the oxidative metabolism of pyruvate had been blocked. Measurement of enzyme-activities in cell-free lysates of treponemes kept at intermediate and high DOC showed that similar functional levels of dehydrogenases were present. However, the possibility to decarboxylate pyruvate was lost at high DOC. The decarboxylation reaction was shown to be catalyzed by an inorganic phosphate dependent and coenzyme A independent enzyme, pyruvate oxidase (38). The decarboxylation proceeded with oxygen consumption and hydrogenperoxide was produced. The end product of the reaction was acetylphosphate. The enzyme acetate kinase which was shown to be present in the cell-free lysate, could account for the production of acetate by transferring a phosphate group to ADP. The transfer of the acetyl group to coenzyme A is possible as was shown by the presence of the enzyme phosphotransacetylase (38).

Anabolism of the treponemes is also dependent on the presence of oxygen. Incorporation of radiolabeled precursors into treponemal macromolecules was low or absent under strict anaerobic conditions. This has been shown for uridine (41), amino acids (42), and adenine (44). However, treponemes which were incubated under an atmosphere of air incorporated the 14 amino acids tested into a wide spectrum of treponemal proteins (42). Incorporation of adenine, 2-deoxyadenine and uridine during 6 days was possible when the treponemes were incubated in an atmosphere containing 3 % oxygen in the presence of dithiothreitol as a reducing agent (44). The carbon atoms derived from the catabolized glucose were found in proteins, the pentose sugars of the nucleic acids and in the glycerol portions of phosphatidylcholine and phosphatidylglycerol. No labeled carbon atoms were found in fatty acids (43).

It has been shown that oleate and palmitate were incorporated by the

treponemes, but they were not catabolized by beta-oxidation (37). This property was shared with non-pathogenic treponemes, which lacked, in addition to beta-oxidation, the ability for chain-elongation and desaturation of fatty acids. The fatty acid composition of these non-virulent treponemes was therefore a reflection of the fatty acid composition of the culture medium. This probably also applies to T.pallidum. Mathews et al (45) showed that the fatty acid composition of T. pallidum was quite similar to, although quantitatively different from that of infected rabbit testes.

The possibility that intermediate products of oxygen reduction were detrimental to the survival was investigated by Steiner et al (46). They showed that T. pallidum was sensitive to hydrogenperoxide concentrations which were similar to those to which obligate anaerobes were sensitive. Protective agents against hydrogenperoxide and the hydroxyl-free radical (catalase, peroxidase and mannitol) significantly prolonged treponemal survival in vitro. Catalase (46) and superoxide dismutase (46,47), but not peroxidase (47), were demonstrated in cell-free lysates of treponemes isolated from rabbit testes. However, electrophoretic and immunological studies indicated that they were of host origin and it appeared that treponemes intrinsically lacked such protective enzymes. Taken together, the lack of scavengers for toxic oxygen intermediates could be a main reason for the difficult survival of treponemes outside a host. Besides the susceptibility of important metabolic, SH-groups containing compounds like enzymes and cofactors, Fitzgerald (48) discussed two other mechanisms which might contribute to the in vitro death of T. pallidum. First, the oxidation of unsaturated fatty acids might result in the formation of lipid peroxides in poly-unsaturated fatty acids. The chain reactions that follow might damage the treponemal membranes. Second, the mucopolysaccharides or closely related compounds of the extra-cellular layer of the treponemes might undergo oxidative-reductive depolymerization, which appears to be mediated via free radicals. The attack on the integrity of this layer might also limit the survival of treponemes in vitro.

1.4.2 Adherence of treponemes to cultured cells.

Upon their incubation with tissue culture cells, pathogenic treponemes attach to the surface of these cells (49,50). The attachment of T. pallidum occurred in all the 19 cultured cell types that were investigated. In all cases treponemal motility and virulence was prolonged (49). The attachment was not influenced either by the number of times the cultured cells had been passaged or by the use of stationary or actively growing cell

cultures. Pathogenic treponemes which were killed by heat or air did not adhere. None of the 11 non-pathogenic strains of treponemes investigated adhered to any of the cultures cell types used (51). The pathogenic treponemes adhered to the cell surface via one or both tapered ends. The number of adhering treponemes per cell was dependent on the size of the inoculum and on the incubation temperature. Adherence increased when the temperature was raised from 4°C to 37°C (50). T. pertenue adhered in a similar manner, although smaller numbers per cell were noted (52).

The manner of adherence suggested the presence of specialized structures on the nose-pieces of the treponemes (50). Incubation of [^{35}S]methionine labeled, solubilized T. pallidum with HEp-2 cells and the subsequent solubilization of these cells revealed the presence of three radiolabeled compounds on SDS-PAGE gels with molecular masses of 89.5, 37 and 35 kD (53,54,56). The immunogenic properties were shown by blotting with rabbit immune serum and their origin from the treponemal surface was established by lacto-peroxidase catalyzed radioiodination. These three fibronectin binding ligands yielded a common polypeptide of 12 kD on papain digestion. Antibodies to this polypeptide reduced the treponemal adherence to HEp-2 cells by approximately 50 % of the control values (56).

The adherent treponemes did not exhibit a preference for specific areas on the individual cultured cells but, rather, were randomly distributed over the entire surface. The avid binding of fibronectin to the surface of washed, motile treponemes, the presence of fibronectin in the extra-cellular matrix of the HEp-2 cells and the tip-oriented attachment of the treponemes to glass-surfaces coated with fibronectin suggested a role of this protein in their adherence. Moreover, the adherence of the treponemes to HEp-2 cells was almost completely abrogated by pre-incubation of these cells with anti-fibronectin antibodies. Three proteins with molecular masses of 89.5, 37 and 35 kD from [^{35}S] labeled solubilized T. pallidum were retained on fibronectin-sepharose affinity columns (54). These proteins were of the same size as those previously implicated as treponemal ligands in the attachment process (53). However, the binding of treponemes to the host constituents laminin, collagen types 1 and 4 and the mucopolysaccharide hyaluronic acid has also been demonstrated (57). Fitzgerald et al. (58) demonstrated that treponemes harvested after 7 days had an increased capacity to bind fibronectin as compared to those harvested after 14 days. The larger fibronectin-binding capacity of seven day treponemes was reflected in the retention of 50 % of these treponemes on fibronectin coated glass-wool

columns. This, together with differences between fibronectin adhering and non-adhering treponemes with respect to cellular attachment, in vitro survival and virulence were interpreted as evidence for the existence of subpopulations of treponemes. A number of 3100 fibronectin-binding sites per treponeme has been calculated with an average association affinity constant of 1.85×10^{7} M^{-1}, which is low in comparison to the similar constant for Staphylococcus aureus (5.6×10^{9} M^{-1}). Low affinity was demonstrated by the displacement of the majority of bound radiolabeled fibronectin by the excess unlabeled fibronectin (59). Similar results with respect to the magnitude of the association constant, the number of binding sites and the reversibility of the binding of fibronectin were shown by Baughn (60). However, Thomas et al (55) estimated the number of binding-sites at 4.3×10^{6} per treponeme. They demonstrated further that the cell-binding domain of fibronectin was involved in the binding of fibronectin to the treponemes. The reversible binding of fibronectin would account more readily for the dissemination of treponemes via the body fluids containing excess of fibronectin without blocking all treponemal binding sites for the adherence to host tissues (59).

The adherence of treponemes to host cells is considered to be an excellent aid for the location in the host tissues and to establish infection. Intervention in this interaction could prevent the infection. The capacity of sera from syphilitic rabbits to interfere with treponemal adherence in vitro has been investigated by several authors. Fitzgerald et al (51) showed that rabbit immune sera produced a reduction of 73-100 % in the adherence 24 h after isolation as compared to control sera. On the day of isolation no differences were observed between the effects of syphilitic and control sera. Hayes et al (50) demonstrated a correlation between the FTA-ABS titer of the sera and their capacity for adherence inhibition. The maximum inhibition obtained was 50 %. In their study on the development of adherence-inhibition factors in rabbit sera Wong et al (61) showed that a maximum adherence inhibition of 70 % was attained at day 30 after inoculation as compared to pre-infection sera. This was well beyond the time at which the serological reactions were positive. Most authors have suggested that the factor(s) that inhibit the adherence were antibodies (50,51,61). However, no proof is as yet available to confirm this suggestion.

The interaction of pathogenic treponemes with cultured cotton tail rabbit epithelial (SF1Ep) cells has led to the successful in vitro multiplication of T. pallidum. A careful examination of the optimal oxygen requirements and the appropriate selection of fetal calf serum, that maximally sustained treponemal

survival were imperative. A 100-fold increase in the number of treponemes was obtained after a 12-days co-cultivation period. The retention of virulence was demonstrated by intra-cutaneous injection in rabbits (62). These results have been confirmed by others (63,64). Subculturing has been succesfully performed, but was only possible after a short (3 days) primary culture (65).

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CHAPTER 2.

SYPHILIS.

2.1. Syphilis in humans.

2.1.1. Course of the infection

Once humans are infected with venereal syphilis, which occurs after close physical, mostly sexual contact, the disease can take a capricious course. This has been reviewed in two previously published dissertations (1,2). However, in the classical course of the disease a number of circumscript clinically manifest stages are distinguished. These stages are separated from each other by latent periods of varying length. The first stage after infection is characterized by the presence of the primary complex consisting of one or more firm, painless ulcers and the presence of swollen inguinal lymph nodes. The presence of T. pallidum in these ulcers confirms the diagnosis of primary syphilis. After a latent period, the primary stage is followed by the secondary stage. In this stage the disease is generalized and the patients show characteristic skin lesions and generalized lymphadenopathy. In biopsies of these lesions treponemes can be demonstrated by special staining techniques. The secondary stage is followed by a latent period which can last for several decades. However, in a number of cases the disease manifests again after a varying latent period and reaches a tertiary stage (1). According to the only available source on this subject, in the Oslo study of untreated syphilis, approximately 30 % of the untreated patients enter the tertiary stage. A large variety of organs may be affected in this stage. The most frequent affections are cardio-vascular syphilis and neurosyphilis. It was estimated that syphilis is the primary cause of death in approximately 10 % of the untreated patients (3).

2.1.2. Histopathology

2.1.2.1 Primary syphilis.

The first clinical symptom of syphilitic infection is the formation of a small papule at the site of inoculation. Histologic examination of biopsies reveals the presence of central deposits of mucopolysaccharides containing a few inflammatory cells. This area is surrounded by an infiltrate consisting of

polymorphonuclear leucocytes, lymphocytes and macrophages. When the papule evolves to an ulcer, a histologically central necrosis is present. In the upper layer of the dermis, a dense infiltrate is seen, in the deep layer and at the periphery of the lesion the infiltrate surrounds the blood vessels. The infiltrate consists predominantly of lymphocytes, plasma cells and macrophages. Characteristically, endothelial cell proliferation is present and there is a thickening of the vessel walls. Treponemes, often in large numbers, can be detected by silver impregnation methods. They are located in the vessel walls, around the vessels and the dermo-epidermal junction at the periphery of the lesions (4).

2.1.2.2 Secondary syphilis.

Secondary lesions have clinically and histologically a varied appearance. The most common histological changes are proliferation and swelling of the endothelial cells and the presence of a perivascular infiltrate. In the macular lesions only minimal histological changes occur. The aberrations of the vessels are restricted to the superficial layers of the dermis. There is a limited endothelial swelling and a limited perivascular infiltrate. In other types of secondary lesions similar aberrations are present on a larger scale with perivascular cuffing around the blood vessels in the superficial and deeper layers of the dermis. Sometimes the dermo-epidermal junction is obscured by a massive infiltrate. The nature of the inflammatory cells varies considerably from case to case. In the classical form, the infiltration by plasma cells predominate. However, frequently a mixed infiltrate is present, consisting of macrophages, lymphocytes and polymorphonuclear leucocytes. Each of these cell types may predominate. The number of treponemes in the secondary lesions shows a correlation with the type of skin lesion. In the macular type the treponemes are usually not detected by specific staining methods. In the papular lesions treponemes are usually present in limited numbers, especially in the dermo-epidermal junction and between the cells of the lower epithelial layers. Condylomata lata harbour large numbers of treponemes (4,5).

2.2. Syphilis in rabbits

2.2.1. Course of experimental infection.

The most frequently used strain for experimental infection is the pathogenic Nichols strain of Treponema pallidum. This strain was isolated from the spinal fluid of a patient with neurosyphilis by Nichols in 1912 (6) and

adapted to rabbits. Since then it has been propagated in rabbits.

Upon intratesticular inoculation the rabbits develop orchitis. The period needed depends, among other factors, on the number of treponemes in the inoculum. Usually a 10-fold difference in number of treponemes results in a difference of 4 days in incubation time (7). Development of orchitis is accompanied by a considerable increase in the weight of testes (8,9). After some time orchitis subsides and the size of the testes is reduced.

The first sign after intracutaneous inoculation on the shaven back of rabbits is the formation of a small papule, appearing after a few days to several weeks, depending on the size of the inoculum. The same dose dependence as mentioned above applies. The papule increases in size during the next ten days to a final diameter of 1.0 to 1.5 cm. Subsequently, it becomes indurated and necrosis and ulceration appear. Next, the lesions regress and heal with or without residual scaring (7).

Following inoculation of rabbits by either of the two routes, there is a rapid dissemination of treponemes. The microbes have been recovered from the blood stream after periods varying from minutes to a few hours (10,11). Generalized lesions of skin and bones occur with a preferential localization in areas with a somewhat lower surface temperature than the general body temperature (7).

Intravenous inoculation results in a large number of disseminated skin lesions after 3 to 8 weeks. It has been used by some authors as the best available animal model for secondary syphilis (7,68).

After the initial and generalized lesions have subsided, no external sign of infection remains. Nevertheless, infectious treponemes have been recovered from lymph nodes and blood of rabbits several years after the disease had become asymptomatic (7,12,25).

A stage parallel to the tertiary stage of syphilis in humans does not occur in rabbits.

2.2.2. Histopathology of experimental syphilis.

The histological picture of a syphilitic lesion is a mixture of several responses which almost always overlap and which may be individually inconspicuous in any single lesion (7). It is convenient to distinguish three stages in the total reaction; a) the inductive stage which comprises the changes produced by the accumulation of treponemes and characterized by the presence of a mucoid material; b) the reactive stage in which the changes following the immune reaction are characterized by an infiltration of

mononuclear cells; and c) the latent stage in which the changes associated with necrosis are followed by the processes of inflammation and repair. This applies to both intratesticular and intradermal infections (8).

The first histological change noted was the deposition of an amorphous, acellular, metachromatically staining substance between the fibres of the connective tissue. Treponemes were preferentially located in the amorphous areas (16). The metachromatic character of the amorphous substance on staining with toluidine blue and related stains and the digestion of this substance with hyaluronidase indicated that acid mucopolysaccharides or closely related substances accumulated during infection (16,17).

After intratesticular infection, the first signs of atrophy of the seminiferous tubules were accompanied by a lymphocytic infiltration of the interstitial spaces. This infiltration increased until maximum orchitis. Lymphocytes predominated but macrophages were also observed. Nearly all the infiltrating lymphocytes stained with a polyclonal antiserum to rabbit thymocytes, identifying them as T-lymphocytes. Only a few B-lymphocytes were observed (18). However, Wicher and Wicher (19) observed on the third day small areas, displaying acute orchitis among morphologically intact testes tissue. In these areas the infiltrate consisted of PMN's (75 %), histiocytes (15 %), lymphocytes (10 %) and occasional plasma cells.

The number of treponemes increased gradually until maximum orchitis and then decreased drastically after the testes became infiltrated with large numbers of macrophages. The drop in the number of treponemes was presumably caused by their phagocytosis by macrophages, since fluorescent parts were observed inside these cells (18). These data were confirmed by ultrastructural analysis. Treponemes were found in phagocytic vacuoles. Their destruction was demonstrated by swelling and lysis. The coating of the treponemes and the inflammatory cells with the amorphous ruthenium red stainable substance did not inhibit the phagocytic process. In fact, the phagocytic vesicles were lined on their inside with this substance (20). These results point to a strong immunological reaction with the characteristics of a delayed hypersensitivity reaction which eradicates most of the microbes (21). However, it was demonstrated that after intradermal infection, small numbers of treponemes persisted at the inoculation site and in distant tissues. Treponemes were observed sporadically in the lymph nodes, the spleen, the testes and at the inoculation site in the dermis. In the testes, this was not accompanied by an inflammatory reaction and no lymphoid cell infiltration or effusion of immunoglobulins was observed (21).

During the reactive stage there was a strong follicular hyperplasia in the lymph nodes and the spleen and a diffuse hyperplasia of the cortical areas in the lymph nodes and peri-arteriolar areas of the spleen (22).

An interesting phenomenon concerning the composition of the infiltrate was observed during the Jarisch-Herxheimer reaction in rabbits. Rabbits inoculated intradermally with treponemes were treated with penicillin and skin biopsies were taken just before treatment and at regular intervals during two days after treatment. The infiltration in the pretreatment biopsies showed an accumulation of lymphocytes, plasma cells and histiocytes. Only a small number of PMN's were present. This number more than doubled within 4 h after treatment and decreased slowly after 24 h (23).

2.3. Development of immunity to syphilis.

2.3.1 Immunity in patients.

In humans, the data on the development of resistance to re-infection stems from the Sing Sing study (24). Volunteer patients with a known stage of syphilis were inoculated with the pathogenic Nichols strain treponemes. Patients with untreated latent syphilis showed no clinical or serological changes after challenge. Half of the patients who had been treated for a proven or highly probable late latent syphilis showed no clinical or serological changes after challenge. Patients, who had been previously treated for early syphilis showed an increase in the titers of antibodies and the majority developed darkfield positive lesions. This study demonstrated that a long existing, untreated infection provided protection against re-infection.

2.3.2 Immunity in rabbits.

Injection of a single dose of treponemes to rabbits resulted after approximately 3 months in a situation, that upon intracutaneous challenge no lesions developed. When challenged before three months the rabbits developed delayed, less severe lesions without ulceration. No treponemes could be demonstrated in these lesions. The occurrence of these phenomena are proportional to the duration of the infection. Termination of infections of less than 3 months duration resulted in a similar partial protection against re-infection. Penicillin treatment 3 months after infection did not change the state of protection. These results show, that infections with T. pallidum in rabbits led to protection, albeit that a rather long period is required (7).

Although these results showed that in patients as well as in rabbits a resistance to re-infection develops, some reservations have to be made. It turned out that immunized rabbits, despite this resistance, harbour infectious treponemes in different tissues. The infectiousness of these treponemes was demonstrated by the injection of extracts of these tissues into fresh rabbits, which subsequently developed syphilitic infection (7,12). Similar results were also obtained when extracts of tissues from clinically cured patients were used (13,14,15). It is unknown how these treponemes survived in a host, which was resistant to challenge. For this reason, this state of immunity is referred to as chancre immunity (7). Although the vast majority of the treponemes occur in the host extracellularly, some authors have observed treponemes which were located intracellularly using electron microscopy (26-29). It has been suggested that the treponemes survive in this manner by withdrawing themselves from the immunological defense mechanisms. However, the virulence of such treponemes has as yet to be demonstrated.

2.4. Immunization.

2.4.1. Active immunization

Despite the development of resistance during the course of the experimental disease in rabbits, most of the attempts to induce immunity by vaccination have failed. Injection of T. pallidum inactivated by lyophilisation, heat, merthiolate or arsenicals or the injection of protein antigens or ultrasonic lysates of T. pallidum or of related non-pathogenic treponemes offered no protection against symptomatic infection (30). However, some vaccination attempts protected rabbits against symptomatic infection, but the protection against asymptomatic infection was only partially achieved (31). The only successful vaccination which offered protection against symptomatic as well as asymptomatic infection was performed by Miller (33). Twenty four rabbits were immunized intravenously over a 37 week period with a total of 3.71×10^9 freshly harvested T. pallidum, which were rendered non-infectious by gamma-irradiation from a ^{60}Co source. All 24 rabbits were observed to be completely resistant to intradermal challenge with doses of upto 10^5 treponemes. This immunity persisted for at least one year. This was demonstrated by the absence of lesions upon re-challenge and by the absence of infection in rabbits which were injected with extracts of lymphnodes and testes obtained from immunized and challenged rabbits.

In Miller's experiments a low reactivity was observed in 46 % of the

rabbits in the VDRL-test, in 91 % of the rabbits in the FTA-ABS test and in 18 % of the rabbits in the TPI tests. These percentages and the range of the titers did not change when the rabbits were challenged. Engel et al (32) observed that the immunization procedure resulted in low titers in the TPHA-test only, FTA-ABS- and TPI reactive antibodies were absent. Challenge of successfully immunized rabbits caused only a slight increase in TPHA- as well as TPI reactive antibodies during the observation period of 5 months. In rabbits with unsuccessful immunization and in control animals, the challenge resulted in comparable high titers of antibodies in these tests. Metzger et al (31) observed no correlation between the degree of TPI reactivity after the immunization procedure and the degree of protection against challenge. Taken together, these results suggest that antibodies detected by these tests are not associated with the development and the persistence of immunity.

2.4.2. Passive immunization

In 1973 three reports were published on the protection of rabbits against challenge by passive transfer of immune sera (34,35,36). Although varying amounts of immune sera were administered either by the intravenous or intraperitoneal route, the results showed that all the sera protected rabbits developed lesions. However, the lesions in these animals differed from those in the control animals in the following manner; a prolonged incubation period, lesions were less severe, the failure to demonstrate treponemes and a faster healing. Moreover, in one report disseminated lesions occurred in 18 % of the protected rabbits as compared to 81 % in the control rabbits (35). In none of these three reports protection against asymptomatic infection was investigated. However, Sepetjian et al (36) concluded that such protection was not obtained since the titers of antibodies in serological reactions, after an initial decrease, started to increase once the treatment with immune serum was stopped.

The protection of rabbits against challenge by series of intravenous or intraperitoneal injections of immune serum was investigated by Bishop and Miller (37) and Weisser et al (38). In both reports protection against symptomatic infection during the period of immune serum administration was observed. In Miller's experiments the rabbits developed flat, pale, non-ulcerative darkfield negative lesions after immune serum injections were stopped. This type of lesion is considered as a characteristic of partial immunity. However, the time lapse between the cessation of the immune serum administration and the development of lesions in the immune serum recipients

was similar to the period needed for the development of lesions in the unprotected control rabbits. Protection against asymptomatic infection was not observed. Virulent treponemes were isolated from tissues of the 4 surviving immune serum recipients. This was demonstrated by the syphilitic infection caused in fresh rabbits (37). Titus and Weisser (39) have provided evidence that IgG from the immune serum was responsible for the protection.

2.4.3 Induction of non-specific resistance.

After the demonstration that intravenous infection with T. pallidum conferred immunity in rabbits against challenge with Listeria monocytogenes Schell and Musher (40) concluded that the infection with T. pallidum led to activation of T cells and macrophages. Subsequently, it was investigated whether an antigen that stimulated cellular immunity would change the course of T. pallidum infection. However, rabbits that were treated intravenously with BCG and showed resistance to Listeria monocytogenes infection, were not protected against intravenous challenge with T. pallidum (41). Similar results were obtained by Graves and Johnson (42) in rabbits which were challenged with intradermal or intravenous injections of BCG. An additional second intradermal treatment with BCG at the same time and at the same site as the T.pallidum challenge failed to alter the course of the development of syphilitic lesions. However, Hardy et al (43) noted a faster enlargement and an earlier onset of healing of primary lesions in BCG sensitized rabbits that were treated with intradermal injection of a mixture of BCG and T. pallidum. Histologically, the lesions in BCG sensitized animals contained a larger number of activated macrophages and a smaller number of treponemes.

2.5 Adaptive immunity to syphilis.

2.5.1 Humoral immune response.

2.5.1.1 In patients.

IgG, IgA, IgM (1,44) and IgE (45) levels were all increased in the sera of syphilis patients. During the earliest diagnostic stage there was already an increase in the serum levels of the three major immunoglobulin classes. IgM and IgA levels reached their maximum at the end of the primary stage, IgG levels did so during the secondary stage. In the early latent stage, IgA and IgG levels did not differ significantly from those observed in control

individuals. IgM levels were still significantly increased (1). The presence of anti-treponema antibodies of the three major immunoglobulin classes was demonstrated in the FTA-ABS reaction during early as well as late syphilis. (46,47). Bos (48) showed that sera from patients with early and late syphilis contained FTA-ABS reactive IgE class antibodies.

2.5.1.2 In rabbits.

No studies have been performed on the levels of serum immunoglobulins during a syphilitic infection in rabbits. However, from experimental infections it is clear that rabbits produce anti-cardiolipin as well as anti-treponemal antibodies. The titers show a similar course as observed in patients. In a small group of rabbits it was observed that the diagnostic tests that were used at present have the same sensitivity in detecting the earliest stage of infection as they did in patients (49). The role of antibodies in the attempts to immunize rabbits and in conferring passive resistance to experimental infection has been reviewed in section 2.4.

The local production of antibodies in the testes of infected rabbits was analyzed by Wicher and Wicher (50). The testicular fluids from infected rabbits at maximum orchitis showed an increased IgM index as compared to controls. No differences were noted in the IgG index. Anti-treponemal IgM titers were slightly higher than those in the serum while IgG titers in the testicular fluid were lower. Although increased leakage of serum proteins across the blood-testes barrier was present, these authors concluded that anti-treponemal IgM was produced locally, but that the local IgG producing plasma cells had a lower production capacity or were under a selective suppressive effect or both.

2.5.1.3 Treponemal proteins detected by antibodies in human sera.

The solubilization of treponemes and the subsequent separation of polypeptides on polyacrylamide gels have been used to identify treponemal antigens. On gels stained with Coomassie Blue upto 35 polypeptides with molecular masses between 14 and 100 kD have been identified (51). Immunoblotting studies demonstrated that a limited number of polypeptides were reactive with IgG and IgM class antibodies in the sera from patients with primary syphilis (52,53,54). Hanff et al (52) distinguished a set of 6 polypeptides, termed major antigenic proteins (MAP), of molecular masses of 45, 33, 30, 15.5, 42 and 16.5 kD. Sera of patients with primary syphilis contained IgM and IgG antibodies to these MAP with the exception of the 42 and

16.5 kD polypeptides. In addition, these sera contained IgM antibodies to a few other polypeptides of varying size. The 42 and 16.5 kD polypeptides were considered as markers for non-primary syphilis. The sera of patients with secondary and early latent syphilis contained IgG antibodies to MAP and a set of 16 other polypeptides. In the sera of patients with cardio-vascular and late latent syphilis IgG antibodies against MAP and to 4 and 5 additional polypeptides respectively were present. Moskophidis and Muller (54) recognized IgM antibodies reactive with 6 polypeptides of molecular mass 69, 38.5, 37, 35, 33 and 30.5 kD and IgG antibodies reactive with 37, 33 and 30.5 kD polypeptides in the sera of patients with primary syphilis. The sera from patients with secondary syphilis contained IgM antibodies reactive to 23 polypeptides of molecular mass ranging from 13.5 to 200 kD. IgG antibodies in these sera reacted to polypeptides of molecular masses ranging from 15.5 to 115 kD.

2.5.1.4 Treponemal proteins detected by antibodies in rabbit sera.

Using immunoblotting and immunoprecipitation techniques it was observed that IgG antibodies to treponemal antigens develop steadily during syphilis infection in rabbits (55,56). A weak IgG response to two polypeptides (60 and 46 kD) was detected three days after intratesticular infection. As the infection progressed IgG antibodies to a steadily increasing number of polypeptides were detected until day 30, when IgG antibodies reactive to all polypeptides were observed. (55). However, the first IgG response that was observed on day eleven post-infection by Stamm and Bassford (56) was against a single 40 kD polypeptide. All treponemal polypeptides were precipitated by IgG on day 35 post-infection. Evidence was presented that the 40 kD polypeptide was a part of the IgG reactive protein most probably derived from the treponemal outer membrane. Interestingly, these authors identified small extracellular antigenic polypeptides in the supernatants of labeled treponemes. Evidence was presented that these proteins were actively secreted by the treponemes and not a product of their lysis.

2.5.2 Immune complexes.

2.5.2.1 Patients.

Renal disease is a well recognized though an uncommon complication of secondary syphilis (57-62) and congenital syphilis (63-66). Renal disorders have been shown to result from immune complex depositions. The participation

of the anti-treponemal antibodies in the formation of these complexes was demonstrated by the positive serological reactions in the material isolated by acid elution of renal biopsies (61). The treponemal constituents were demonstrated by the positive immunofluorescence with immune rabbit serum on the renal biopsy from a patient with secondary syphilis (62). Solling et al (67) analyzed the presence of circulating immune complexes (CIC) in the sera of patients with syphilis. Using the Clq binding assay, they showed CIC in the sera of 6 out of seven patients with secondary syphilis. In the sera of 4 patients with primary and 2 patients with neurosyphilis no CIC were detected. Engel and Diezel (68) used polyethyleenglycol precipitation and demonstrated CIC in one out of 6 patients with primary syphilis and in 20 out of 45 patients with secondary syphilis. In the latter group the number of positive patients was reduced by 50 % by treatment with penicillin. The antibodies present in the rinsed and solubilized immune complexes were reactive in serological tests for syphilis.

2.5.2.2 Rabbits.

Baughn et al (69) demonstrated that 86 % of the rabbits that were infected intravenously developed disseminated skin lesions and circulating immune complexes. These complexes were present in 50 % of the rabbits inoculated intradermally but were absent after intratesticular infection. The CIC in the sera of intravenously infected rabbits had sedimentation coefficients greater than 19 S and contained IgG, IgM and C3 (70). The treponemal constituents of these complexes were demonstrated by their reactivity to sera from rabbits which were immunized with T. pallidum. Electroblots of purified CIC showed that at least 8 different treponemal constituents could be identified with rabbit immune sera. Antibodies obtained from isolated CIC reacted with the same treponemal constituents using electrophoretically separated treponemal polypeptides. Moreover, antibodies to these polypeptides were present in the sera of rabbits immunized with purified CIC (71,72).

2.5.3 Humoral immune response to unrelated antigens.

The humoral immune response to the unrelated T cell dependent antigens was investigated in rabbits which were injected intravenously with 2×10^9 sheep red blood cells (SRBC) at various intervals after intravenous infection with T. pallidum. After a week, their spleens were assayed for the number of direct (IgM) and indirect (IgG) plaque forming cells (PFC) to SRBC. The number of IgM PFC increased in syphilitic rabbits during the period of incubation and overt

disease as compared to control rabbits which were injected with SRBC alone. This number returned to normal after the cutaneous lesions had healed (8 to 10 weeks). To the contrary, the number of IgG PFC diminished progressively during the incubation period and reached the lowest levels during overt disease and returned to normal when the lesions had healed. Penicillin treatment of the syphilitic rabbits before the administration of SRBC resulted in normal levels of both types of PFC (73). A suppressor cell population, that inhibited the secondary response was observed among the peripheral blood lymphocytes of control rabbits which were sensitized to SRBC and boostered after 6 to 9 months. Such a suppressor cell population was absent in syphilitic rabbits which were treated in the same manner (74). Using inbred control rabbits pre-sensitized to SRBC, it was shown that the secondary IgM and IgG PFC response of spleen cells was inhibited by spleen cells or lymph node cells from syphilitic rabbits. No special inhibiting cell population was observed among the nylon wool adherent or non-adherent spleen cells or lymph node cells. However, the inhibition was abrogated by treatment of the inhibiting cells with proteolytic enzymes, but not with hyaluronidase. Moreover, the inhibiting effect could be transferred by the cell-washings of these cells. The sera of syphilitic rabbits which contained circulating immune complexes, inhibited the plaque forming assay in a dose dependent manner. No correlation was observed between the amount of mucopolysaccharides in the syphilitic sera and the degree of inhibition they could produce (72).

2.6 Cellular immune response.

2.6.1 Skin tests in patients.

The skin tests performed by intracutaneous injection of purified, preserved Nichols treponemes has been re-evaluated as a diagnostic aid in sero-negative late cases or as an index of disease activity in sero-positive cases without clinical lesions. The clinical and histologic pattern showed the characteristics of a delayed type hypersensitivity reaction (75,76). The results indicated that control individuals and patients with primary syphilis always showed a negative skin reaction to this antigen, whereas patients with secondary and early latent syphilis were occasionally positive. About half of the patients with late latent syphilis and the majority of patients with tertiary syphilis showed positive skin reactions (76,77,78). Wright and Grimble (78) interpreted these results in retrospect as evidence for a suppressed cellular immunity in early syphilis.

A considerable amount of literature exists on the demonstration of a possible suppression of lymphocyte functions in vitro. Since lymphocytes as well as factors, present in the sera of patients and infected animals have been implicated, these reports have to be evaluated accordingly. The presence of an intrinsic effect of the infection on the lymphocyte functions was judged from lymphocyte cultures using control sera. The presence of suppressive factors in the sera of syphilis patients and syphilitic rabbits were evaluated by culturing lymphocytes from healthy subjects in their presence.

2.6.2 Mitogenic stimulation.

2.6.2.1 Patients.
No studies are available.

2.6.2.2.Rabbits.

In experimental syphilis conflicting results were obtained. Pavia et al (79,80) observed a diminished response of peripheral blood lymphocytes of syphilitic rabbits to Con A, PHA and PWM as compared to their response to these mitogens before infection. These authors also found a diminished response to PHA and PWM in suspensions enriched for T cells by passage through nylon-wool colomns as compared to similarly treated T cells from uninfected rabbits (81). However, Baughn and Musher (82) did not find such a difference in the results of mitogen stimulation of peripheral blood lymphocytes from infected and uninfected rabbits.

2.6.3 Suppression of mitogenic stimulation by serum factors.

2.6.3.1 Patients.

Levene et al (83) showed that the PHA response of lymphocytes from a single healthy donor, cultured in the presence of the sera from patients with secondary syphilis, was reduced as compared to that in the presence of autologous serum. The sera of patients with primary or latent syphilis lacked this capacity. Thompson et al (84) showed that 20 % of the sera from patients with primary and 44 % of the sera from patients with secondary syphilis were capable of suppressing slightly the PHA stimulation of peripheral blood lymphocytes from healthy subjects. However, Musher and Schell (82) and Fromm et al (85) were not able to demonstrate a plasma- or serum-mediated suppression of the PHA response of control lymphocytes.

2.6.3.2 Rabbits.

Ware et al (86) showed that 71 % of the sera from T. pallidum infected rabbits were capable of reducing the response of peripheral blood lymphocytes from healthy donors to Con A. This effect was present 7 days after infection and it reached its maximum at peak orchitis and remained present until approximately 2 weeks after infection. Wicher and Wicher (87) obtained similar results using PHA. Ware et al (88) demonstrated that the serum-mediated reduction in Con A response was attributable to the participation of only a part of the Con A sensitive lymphocyte pool. Bey et al (89) demonstrated that addition of the serum or the testicular fluid (accumulating between the blades of the tunica albuginea at orchitis) from infected rabbits to cultures of normal peripheral blood lymphocytes in homologous serum diminished the Con A response strongly. It was shown that both the serum and the testicular fluid contained mucopolysaccharides, which could not be demonstrated after treatment with hyaluronidase. This treatment also destroyed the suppressive effect of these fluids for the response to Con A stimulation. These results were confirmed by Baughn and Musher (90). The sera and testes supernatants of T. pallidum infected animals suppressed the Con A response when added to these cultures. Immune complexes, isolated from these sera and testes supernatants did not influence the results of Con A stimulation, in contrast to isolated mucopolysaccharides. The suppressive capacity of these fluids disappeared after filtration through Sepharose-Con A columns, suggesting a direct interaction between the suppressive substances and Con A. This suggest that active sites on the mitogen are covered by these substances, resulting in a diminished recognition by functionally intact cells.

2.6.4 Response to antigens.

2.6.4.1 Patients.

Friedman and Turk (91) demonstrated a "spectrum of lymphocyte responsiveness" upon stimulation of peripheral blood lymphocytes of patients from various stages of syphilis. Lymphocytes from patients with sero-negative primary syphilis showed no increased blastogenesis when stimulated with whole treponemes. Lymphocytes from patients with sero-positive primary syphilis showed a significantly higher response. The same was true for lymphocytes from patients with the papular form of secondary skin lesions. Lymphocytes from patients with the macular form of secondary syphilis demonstrated a decreased

response to T. pallidum antigens. Antibiotic therapy caused a greater increase in the stimulation by T. pallidum in all patients with sero-positive primary and secondary syphilis.

2.6.4.2 Rabbits.

The response of peripheral blood lymphocytes (PBL) and lymphocytes derived from spleens and lymphnodes to treponemal antigens has been shown to increase gradually during the initial stages of the infection and eventually reach a plateau level. Using PBL, the blastogenic response was shown to start 3-4 weeks after infection. When lymphocytes isolated from the spleen were used, the first blastogenic response was noted 6-10 days after the inoculation (92). At the end of the experiments (12 weeks) increases of response upto 600-fold were observed as compared to the response of spleen cells from uninfected controls (93). The blastogenic response of T cell enriched suspensions from PBL and spleen cells showed increased levels of response than their respective unseparated suspensions (93,94). The response of the T cell enriched suspensions was completely inhibited by the treatment of these cells with a polyclonal T cell antiserum and complement (93).

2.6.5 Suppression of antigenic response by syphilitic serum.

2.6.5.1 Patients.

No studies in patients are available.

2.6.5.2 Rabbits.

In several reports it has been shown that the antigenic response of lymphocytes from infected animals was reduced in the presence of sera from infected animals. Pavia et al (93) reported that serum from infected rabbits caused 35 % reduction in the response during the first two weeks of infection. At the end of the experiments (12 weeks), this reduction had increased to 56 %. The response of T cell enriched suspensions from PBL to antigenic stimulation was inhibited by approximately 50 % by these sera after the first week of infection and remained at this level until the end of the experiments. Using rabbit immune sera Maret et al (94) reported a 2.5 to 6-fold reduction in the stimulation index of the antigenic stimulation of unseparated as well as T cell enriched suspensions of spleen lymphocytes 10 days after infection. Baker-Zander et al (95) demonstrated that the inhibition of antigenic responses depended strongly on the amount of serum present in the culture

media. Spleen lymphocytes from infected rabbits showed an optimal response to T. pallidum antigens in the presence of 1 % normal rabbit serum. The same amount of syphilitic serum (1 %) in the culture media reduced the response to treponemal antigens by 17- 20 %. However, in the presence of 10 % syphilitic serum, the lymphocyte response was inhibited by 55 % on day 10 and by 80 % on day 31 (end of the experiment). These authors isolated two fractions from sera of syphilitic rabbits that were suppressive. One fraction, containing low-molecular weight substances that were soluble at 50 % ammoniumsulphate saturation, inhibited both the antigenic and the mitogenic Con A response. A second fraction, which contained high-molecular weight substances insoluble at 50 % ammoniumsulphate saturation inhibited only the antigenic response. This second fraction contained alpha-globulins and FTA-ABS reactive IgM. Neither of these two fractions contained immunoglobulins or treponemal components on immunodiffusion. Moreover, IgG isolated from the immune sera did not exert any inhibition effect on the antigenic stimulation of lymphocytes.

2.7 Destruction of treponemes by the host.

Their role as the ultimate effector cells responsible for the elimination of microbes from host tissues needs the activation of the macrophages by specifically sensitized T cells. The production of two soluble products, the leucocyte migration inhibition factor and the macrophage activating factor were investigated in syphilis.

2.7.1 Leucocyte migration inhibition in patients.

The results of the various studies which used peripheral blood lymphocytes are confusing. Fulford and Brostoff (96) measured leucocyte migration inhibition in the presence or absence of the protein antigen from T. phagedenis, biotype Reiter. Leucocyte migration inhibition was only obtained in the six patients with late active syphilis. In patients with primary syphilis, enhanced migration was observed, while in patients with secondary syphilis no differences as compared to the controls were observed. Fromm et al (85) used sonicated T. pallidum as the antigen. They saw no differences between the 9 patients with early syphilis and the controls, but the treatment of the patients resulted in an enhanced spontaneous migration of their leucocytes. Gschnaitt et al (97) investigated a total of 17 patients with different stages of syphilis. They observed no leucocyte migration inhibition when sonicated T. pallidum was used as the antigen. However, a significant

migration inhibition was obtained as early as 2 days after the start of treatment, being most pronounced after one week. After treatment, migration inhibition was still absent in 2 patients with primary syphilis, but all 4 patients with secondary syphilis showed strong migration inhibition one week after the start of treatment. This inhibition lasted for at least one year. Three patients with neurosyphilis and 7 out of 8 patients with latent syphilis showed migration inhibition after treatment. Since all 17 patients showed migration inhibition when Con A was used instead of T. pallidum sonicate as the antigen, the authors concluded that a specific blockage of migration inhibition existed to T. pallidum antigens in patients with syphilis.

2.7.2 Leucocyte migration inhibition in rabbits.

Pavia et al (98) used peritoneal exudate cells from rabbits infected with T. pallidum as indicators of macrophage migration inhibitory activity. When various concentrations of the protein antigen from T. phagedenis, biotype Reiter were used, the migration of these cells was inhibited during the period between 3 and 15 weeks after infection. Wicher and Wicher (99) showed migration inhibition of peripheral blood leucocytes to the antigens of sonicated T. pallidum as early as one week postinfection and lasting for at least 16 months. Using low concentrations of the antigen a biphasic response was measured, a stimulation of migration was noted during the first two weeks, which changed to an inhibition by week 4 (98,99).

2.7.3 Macrophage activation in rabbits.

Lukehart (100) investigated the production of soluble macrophage activating factors by splenic lymphocytes from syphilitic rabbits 5-8 weeks after infection and from control rabbits. Supernatants from cultures of these lymphocytes either stimulated with sonicated T. pallidum or Con A were tested for their ability to inhibit the intracellular multiplication of Listeria monocytogenes in cultures of peritoneal exudate cells from normal rabbits. Supernatants from unstimulated lymphocyte cultures or T. pallidum stimulated cultures of lymphocytes from uninfected rabbits were unable to activate the macrophages. Supernatants from cultures of specifically stimulated lymphocytes from infected rabbits and Con A stimulated lymphocytes from infected and uninfected rabbits were able to activate the macrophages and thus confer resistance to Listeria infection.

2.7.4 In vitro phagocytosis of T. pallidum.

The phagocytosis of motile treponemes by rabbit peritoneal exudate cells was investigated by Lukehart and Miller (101). Using immunofluorescent techniques the presence of treponemes inside the macrophages after a 4 h co-incubation period was observed. Ingested treponemes appeared within the cytoplasm as round fluorescent bodies which increased in number as the co-incubation time increased. Electron microscopic observation showed that the treponemes were located in the typical phagocytic vacuoles. Heat-stable components from the immune sera significantly increased phagocytosis.

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CHAPTER 3.

3.1 Summary of the literature.

The histopathological and ultrastructural studies in experimentally infected rabbits have demonstrated that large numbers of treponemes are destroyed by the host. This was accompanied by a vigorous immune response during which the treponemes were ingested and destroyed by macrophages. However, these studies have also demonstrated that a small number of treponemes persist both at the local inoculation site as well as at distant sites resulting in an asymptomatic infection. The inoculation of rabbits with either the extracts of tissues from patients or rabbits with asymptomatic infection induced the disease demonstrating the pathogenicity of the persistent treponemes. It is unknown how these treponemes escaped from the hosts defence mechanisms. Between 1970 and 1980 many investigations were undertaken in order to demonstrate a suppression of the immune mechanisms in patients and infected rabbits resulting in an incomplete eradication of the treponemes. Since the treponemal antigens used in these in vitro studies originated from the treponemes harvested from infected rabbits, they were poorly defined with respect to their purity. It is also unknown from which treponemal structures were the antigens that evoke reactivity in these assays derived. However, the survey of these studies in Chapter 2 does not support the hypothesis of a generalized immuno-suppression. Sensitization of lymphocytes, notably of the T cells with treponemal antigens had occurred in patients as well as in experimental animals. This was demonstrated by the in vitro stimulation of peripheral blood lymphocytes from patients and lymphocytes from different sources from infected rabbits with treponemal antigens; by the production of soluble factors by lymphocytes which act on the macrophages and by the phagocytosis followed by desintegration of the ingested treponemes by the macrophages in vivo as well as in vitro. Moreover, the syphilitic infection also produced an activation of the non-specific defence mechanisms, as demonstrated by the inactivation of Listeria monocytogenes by rabbits infected with Treponema pallidum. The argument that supports the suppression hypothesis is the occurrence of serum factors which inhibit the mitogenic and antigenic stimulation of lymphocytes. Their importance in this respect is unclear. However, for at least one mitogen (Con A) it has been demonstrated that the acid mucopolysaccharides present in the sera of infected experimental animals bind to and reduce the effective

concentration of this mitogen. Moreover, in patients these suppressive factors are most easily demonstrated during the secondary stage of the disease. During this stage the symptomatic character of the disease can change into an asymptomatic one. It is questionable whether this is compatible with the idea of an effective immune suppression.

Soluble immune complexes, formed when the antigen is in excess, have been shown to exert an immunosuppressive effect. Circulating immune complexes have been demonstrated in the sera of patients with secondary syphilis. The antigen/antibody ratio of these immune complexes is as yet unknown. However, for the same reason as mentioned above, it seems unlikely that during the secondary stage the immune complexes are involved in an effective immuno-suppression. Rather, they seem to arise from the release of many antigens as a result of the elimination of the treponemes. Therefore, an alternative explanation for their presence would seem to be a function in the normal immunoregulatory processes.

Concerning the humoral immune response, raised anti-treponemal immunoglobulin levels, detected by the conventional serological techniques are readily demonstrated in the sera of patients and infected rabbits during the early and late stages of the disease. However, the question remains whether the host produces antibodies which exert a treponemacidal effect in vivo in combination with complement and/or phagocytes. Antibodies may be produced that are irrelevant for protection. This is exemplified by the lack of production of the diagnostically important TPI-, TPHA-, FTA-ABS and VDRL reactive antibodies in the (partially) succesfull immunization experiments of rabbits. Immunoblotting of soluble extracts of treponemes demonstrated that IgG and IgM responses developed slowly in patients and in infected rabbits. In patients full antibody response to the majority of the treponemal antigens was present only as late as the secondary stage. This might indicate that the production of antibodies is blocked against a large part of the treponemal antigens or that the immune apparatus is confronted relatively late with these antigens.

An alternative explanation of the chronic state of the disease might be the capacity of (some of) the treponemes to escape from the host's immune defences. It seems reasonable to assume that a successful immune response to T. pallidum must, at least initially, be targetted at the outer surface of the treponemes. It is unknown whether the sensitization against these surface components does in fact occur. The reason is, again, the obscurity concerning the origin of the various treponemal antigens. Intact treponemes, that are used in serological reactions have to be modified by physico-chemical means or

need long in vitro incubation periods before sero-reactivity can be demonstrated. In both instances, the treponemes are altered and no longer represent the in vivo situation. Several possibilities can be considered to explain the low reactivity of freshly harvested treponemes. The first explanation is that the outer membrane components may be of a low immunogenicity and do not stimulate the immune system. A second explanation is that the epitopes are burried inside the outer membrane leaving only the non-immunogenic components of the outer membrane exposed. A third explanation is that the outer membrane of the treponemes is covered by an extra-cellular layer protecting it from immunological attack.

3.2 Aim of the study.

Based on the assumption that a successful immune response to T. pallidum must, at least initially, be targetted against the outer membrane of the treponemes, the purpose of the study was to gain more insight in the accessibility of this membrane. The in vitro adherence of the treponemes to fibroblasts played a key role in the experimental studies. First, the relevance of the treponemal adherence in the in vivo infection was investigated as a part of histopathological studies. Second, since the adherence occurred at the surface of the treponemes, various factors that influenced the adherence were investigated. Third, the adherent treponemes could be used to study the accessibility of the treponemal outer membrane for anti-treponemal antibodies without the use of fixation. Sera of syphilis patients were used as the antibody source. In these patients the IgG response during the sequential stages of syphilis was characterized in order to exclude an overall immuno-suppression during this disease. Moreover, this characterization enabled the use of well-defined sera and their fractions in later studies.

CHAPTER 4.

IMMUNOGLOBULIN G SUBCLASSES OF FLUORESCENT ANTI-TREPONEMA ANTIBODIES: EVIDENCE FOR A SEQUENTIAL DEVELOPMENT OF SPECIFIC ANTI-TREPONEMA IgG RESPONSES IN PATIENTS WITH EARLY SYPHILIS.

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Journal of Clinical Microbiology, 24, 418-423 (1986)

Abstract

The development of immunoglobulin (IgG) subclass-specific anti-Treponema pallidum antibodies during the course of syphilis in humans was studied with sera from 50 untreated male patients. The patients were divided into 5 diagnosis groups. In the fluorescent treponemal antibody test, which delineates the presence of cross-reacting antibodies, as well as specific antitreponema antibodies, IgG1, IgG2 and IgG3 subclass antibodies were already present during the seronegative primary stage. Specific antibodies, which were detected by the fluorescent treponemal antibody absorbtion test, were first present during the serotype-variable primary stage. These antibodies were almost exclusively of the IgG1 and IgG3 subclasses. In later stages, antibodies of other subclasses were detectable. Titration of IgG1 antitreponema antibodies in three electrophoretically different IgG fractions revealed an asymmetric distribution in these fractions during primary syphilis. The antibodies were largely confined to the most basic fraction during primary syphilis. A sudden change in this distribution was noted between the end of the primary stage and the secondary stage; an even distribution of IgG1 antitreponema antibodies existed in the late latent stage. These findings confirm and extend previous results from our laboratory. The development of antibodies detected by both tests is discussed in terms of a sequential stimulation of the immune system due to the presence of an extracellular layer covering the treponemas or, alternatively, in terms of a suppression of the immune response during early syphilis.

Introduction

The development of immunity in syphilis is still poorly understood. In patients in which the disease had reached latency (18) and in experimental

animals, approximately three months after the initial infection (30), resistance to intradermal challenge with pathogenic treponemas was observed. The degree of protection provided by infections for shorter periods correlates with their duration (18,30). This suggests that for the development of immunity, the disease has to follow its natural course for a rather long period.

The relative contributions of humoral and cellular immune mechanisms to the defence against treponemas in syphilis are unknown. The role of a humoral mechanism has been investigated by the transfer of an immune serum to normal rabbits. Several investigators (20,24,29) observed a delay in lesion development and less severity of the lesions with an absence of ulceration and reported a failure to demonstrate the presence of treponemas in the lesions. Protection against challenge during prolonged periods was obtained by a series of injections with immune sera (3,37). However, in these studies, lesions appeared in at least some of the protected rabbits after cessation of immune serum injections and protection against asymptomatic infection was not obtained (3). Titus and Weiser (28) provided evidence that the active entity was immunoglobulin G (IgG).

The slow development of host defense mechanisms on the one hand and the absence of positive results in certain in vivo and in vitro immunological tests (14,16,26,27) on the other could complement each other and led to the hypothesis that the immune system is suppressed during early syphilis. However, it is not clear whether some of these findings are attributable to either a suppressed or a not yet sensitized immune system. We previously reported evidence for a slow development of IgG responses (33). In the present study, we extended these results by comparing the development of IgG subclass-specific anti-Treponema pallidum antibodies in the sera of patients with various stages of syphilis by using the fluorescent treponemal antibody (FTA) and FTA-absorption (FTA-ABS) tests. Furthermore, it was anticipated that a comparison of the development of nonspecific and specific antitreponema antibodies would provide further information on a possible suppression of the immune system with respect to IgG production during early syphilis.

Materials and Methods

Sera. Serum samples were obtained from untreated male patients, 18 to 73 years old (average age,37 years), and were stored at -70°C until use. The clinical diagnosis of syphilis was made according to the criteria described by Menke et al. (H.E. Menke, Ph.D.thesis, Erasmus University, Rotterdam, The

Netherlands 1975; N.H.L. de Jong, J.J. van der Sluis, J. van Dijk and T.E.W. Feltkamp, Letter,Br.J. Vener.Dis., 54, 283, 1978) and was confirmed by positive darkfield examination or a positive T. pallidum Immobilization (TPI) reaction or both. The diagnosis of primary syphilis was differentiated using four serological reactions (Wasserman-Kolmer Complement Fixation reaction, the Reiter protein complement fixation reaction, the Venereal Disease Research Laboratory test and the T.pallidum immobilization reaction). These reactions were performed as reported before (33). Patients whose sera showed a negative reaction in all four tests were classified as seronegative ($S\ 1^-$), patients whose sera were reactive in all four tests were classified as seropositive ($S\ 1^+$), and patients whose sera were reactive in at least one of the tests (but not in all four) were classified as serotype variable ($S\ 1^{+-}$). Patients with secondary syphilis (S II) showed the typical papular or macular skin rash and their sera were reactive in all four reactions. The incubation times of these stadia were (in weeks): $S\ 1^-$: 3.0 $\pm$ 1.1; $S\ 1^{+-}$: 3.9 $\pm$ 1.1; $S1^+$: 5.9 $\pm$ 1.4 and S II: 12.4 $\pm$ 4.3 (Menke, Ph.D. thesis). The patients with late latent syphilis (LL) had no clinical manifestations of infection, and their case-histories showed no previous history of syphilis or syphilis treatment. In all cases their sera had shown positive results in the T. pallidum immobilization reaction on one or more occasions, whereas the results of the cardiolipin reactions were negative or showed only a low antibody titer. Ten sera from each of the five diagnosis groups were investigated. Patients in whom concomitant disease was diagnosed were excluded from the study. Ten sera obtained from healthy blood donors were used as controls.

IgG fractions. Three IgG-containing fractions, differing in electrophoretic mobility, were prepared from five individual serum samples from each of the diagnosis groups, except the $S1^-$ group, by DEAE-Sephadex ion exchange chromatography. Isolation of the fractions and densitometric control of their charge differences were performed as described previously (33). Fraction 1 contained the most basic IgG molecules, and fraction 3 contained the most acidic. The protein concentrations in the fractions were estimated from the extinction at 280 nm, with human IgG as the standard. The protein content was adjusted to 1 mg/ml before use.

FTA-slides. The following procedures were used for the preparation of FTA-slides. The pathogenic Nichols strain of T. pallidum was used as the antigen. The organisms were extracted in Nelsons medium by our standard procedure (34) from the testes of a rabbit, which had been inoculated 1 week earlier. An equivalent amount (milliliters) of medium was added to the minced

testicular tissue. The extraction was performed by shaking for 45 min under an atmosphere of 95 % N_2 -5 % CO_2. After decantation, the supernatant was centrifuged at 800 x g for 10 min to remove particulate matter. Next, the treponemas were sedimented by further centrifugation at 40.000 x g for 15 min. The treponemas were washed once with phosphate-buffered saline (PBS), containing 0.01 % NaN_3 (wt/vol) and stored in 2 ml aliquots at -70°C. FTA-slides were prepared on Cooke microprint stockslides (Nutacon; CL 100 Cell-Line Associates, Inc., Newfield, N.J.), containing 10 spots per slide. The thawed treponemal suspension was adjusted to 30 treponemas per microscopic field (380 x) and applied to the spots with a wire loop. The slides were air-dried, left overnight, and fixed in dry acetone for 10 min. before storage at 4°C.

Antisera. Antisera against the four human IgG subclasses, raised in sheep, were obtained from the Central Laboratory of the Netherlands Red Cross Blood Transfusion Service, Amsterdam . According to the supplier these antisera were specific for their subclass in the passive haemagglutination assays with erythrocytes coated with IgG of the various subclasses and in passive hemagglutination inhibition assays by IgG of the relevant subclass. The method of their production has been reported (7). The specificity of these antisera has been verified by others with different techniques (15,17,21) including fluorescent methods (31,36). Fluorescein isothiocyanate-labeled rabbit anti-sheep IgG was obtained from the same supplier and used as the conjugate.

Absorptions. Preliminary experiments showed that the antisubclass antisera were reactive with T. pallidum. This reactivity was successfully removed by absorption with T. pallidum Nichols. A pellet of 10^8 treponemas was suspended in 0.2 ml of each of the antisubclass antisera. The mixture was incubated for 30 min. at 4°C, and the treponemas were removed by centrifugation. To minimize nonspecific background staining of IgG2 subclass antiserum and IgG serum fractions, absorption with normal rabbit testes powder, prepared as described by Johnson et al (13) was performed. Approximately 10 mg of testes powder was suspended in PBS and sedimented by centrifugation. The pellet was resuspended in 1.0 ml of the appropriate dilution of the antiserum or serum fraction, and the mixture was incubated for 30 min. at 4°C with occasional shaking. The testes powder was sedimented by centrifugation, and the supernatant was used in further experiments. The Reiter ultrasonic extract, which was used to absorb patient sera and serum fractions in the FTA-ABS test, was obtained from the Dutch National Institute of Health, Bilthoven, The Netherlands. It was

prepared by sonifying 50 g. (wet weight) of T. phagedenis, biotype Reiter, suspended in 1 l. of PBS (pH 7.4), at 0 °C. for 10 min with a Sonifier (Branson Sonic Power Co.,Danburry, Conn.) operated at 100 W. The final sonic extract was obtained from this crude mixture by centrifugation at 5,000 x g for 45 min. As preservative, one ml 40 % formaldehyde was added per 100 ml final sonic extract.

Working dilutions. The antisubclass antisera were used at a dilution of 1:10 in combination with a 1:80 dilution of the conjugate. Although checkerboard titrations of the conjugate and the antisubclass antisera on FTA slides incubated with a 1:5 diluted S II sera showed plateau levels at much higher dilutions (IgG1, 1:80; IgG2, 1:40; IgG3, 1:40 and IgG4, 1:20; in combination with 1:160 diluted conjugate) lower dilutions were chosen to avoid false-negative results due to over-diluted antisera in combination with weak syphilitic sera. The controls (see below) performed on each occasion indicated that this introduced no false- positive results.

FTA-procedure. Sera were tested in a 1:5 dilution with either PBS (FTA-test) or an ultrasonic extract of T. phagedenis biotype Reiter (FTA-ABS). After a 30 min preincubation at room temperature, 20 µl of diluted serum was applied to the spots, and the slides were incubated for 30 min. in a moist chamber at room temperature. The slides were rinsed and then washed with PBS containing 2 % Tween 80 for 30 min. with continuous stirring. The washing fluid was changed twice during this period. After air-drying, 20 µl of diluted antisubclass antiserum was applied to the spots. After incubation, washing and drying as described above, 20 µl of diluted conjugate was applied, and the incubation, washing and drying procedure repeated. Next, two drops of mounting medium, consisting of 1 part PBS and 9 parts glycerol, were applied to the slides. The slides were covered with a cover glass and sealed with nailpolish.

Serum fractions containing 1 mg of IgG per ml were tested in the FTA-ABS procedure in a double dilution series. The titer of each fraction was recorded as the reciprocal of the highest dilution, which gave a 1+ positive reaction. The mean titer and standard deviation were calculated from log2 values. For graphical purposes, these values were converted to dilutions. The experiments were arranged in such a way that only sera or serum fractions from one diagnosis group and one type of antisubclass antiserum were applied to a slide.

Controls. The following controls were used. The positive control comprised antigen incubated with S II serum, anti-IgG subclass antiserum and conjugate. The negative controls were as follows: (i) antigen, incubated with conjugate,

(ii) antigen, incubated with S II serum or appropriate IgG fraction and conjugate, (iii) antigen, incubated with antisubclass antiserum and conjugate, and (iv) antigen, incubated with negative control serum, anti-IgG subclass antiserum and conjugate. In these controls, S II serum and the negative control serum were diluted 1:5 in either PBS or Reiter ultrasonic extract. The negative controls gave consistently negative results.

Microscopic examination. Slides were examined with a Leitz Orthoplan binocular microscope equipped with an epiilluminator. The light source was a xenon XBO 75 lamp; the filter combination used was a BG 38 filter (4 mm) and the filterblock K (Leitz) containing the excitation filters 2 x KP 490 and GG 475 (2 mm), the dichroic mirror TK 510, and a barrier filter K 515. The microscope was equipped with a dark-field illumination (condensor D 1.20) objective 95 x with an adaptable numerical aperture and 4 x occulars. The slides were observed under alternating dark-field and U.V. illumination. The results with UV light were scored as follows: 4+ , Very strong fluorescence; 3+ , Strong fluorescence; 2+ , Definite fluorescence; 1+ , Weak fluorescence; $\pm$, treponemas were recognized without any specific fluorescence; and - , neither specific fluorescence nor treponemas were recognized, but treponemas could be recognized under dark-field illumination. The results were finally recorded as either positive or negative. The $\pm$ and - reactions were considered negative.

Results

The results of the FTA-test are shown in Table 1. It can be seen that IgG1, IgG2 and IgG3 subclass antibodies appeared simultaneously. Within these three subclasses, a minority of the S 1^- stage sera showed positive reactions. In the S $1^{\pm}$ stage, all sera showed strong positive reactions, which persisted during the more progressive stages. The first antibodies that were detectable with anti-IgG4 antiserum were observed in the S $1^{\pm}$ stage in one serum. The number of positive serum samples with this antiserum increased to 7 for the S 1^+ stage patients and to 10 for the S II stage patients. In the LL stage, 9 patients showed a positive IgG4 reaction. All 10 donor sera were negative in both the FTA- and the FTA-ABS tests.

The results of the FTA-ABS test were as follows (Table 1). All sera from S 1^- stage patients showed a negative reaction with the four subclass antisera. In the S $1^{\pm}$ stage, all ten patients showed a positive reaction with anti-IgG1 and 9 showed a positive reaction with anti-IgG3. Only one patient in this group was positive with anti-IgG2. For the S 1^+ stage patients, all sera

Table 1.

Number of patients showing positive results for each of the four IgG subclasses in the FTA and FTA-ABS tests.

IgG subclass	No. of pat's showing positive results for the indicated stage[a] in FTA test					FTA-ABS test				
	S1$^-$	S1^{+-}	S1$^+$	SII	LL	S1$^-$	S1^{+-}	S1$^+$	SII	LL
IgG1	3	10	10	10	10	0	10	10	10	10
IgG2	4	10	10	10	10	0	1	10	10	3
IgG3	2	10	10	10	10	0	9	10	10	6
IgG4	0	1	7	10	9	0	0	0	1	0

[a] Ten patients were tested per stage.

showed positive reactions with anti-IgG1, -IgG2 and -IgG3 antisera. This remained during the S II stage. In the LL stage, strong positive reactions were found in all 10 sera with anti-IgG1. In this stage six serum samples gave positive reactions for IgG3 and three gave positive reactions for IgG2. In the FTA-ABS reaction no positive results were obtained with anti-IgG4 antiserum, except for one S II stage serum.

The results of the FTA-ABS test showed a successive involvement of the various IgG subclasses in the anti-treponema response, which followed the sequence IgG1, IgG3, IgG2 and finally IgG4 in one S II stage patient. This is compatible with a shift of anti-treponema antibodies from the basic type of heterogenous IgG to more acidic type during early syphilis. Accordingly, IgG fractions of different electrical charge were isolated and titrated for their IgG1 anti-treponema antibodies. The initial IgG content of the various fractions was adjusted to 1 mg/ml before titration. The results of these titrations are shown in the Fig. 1. It can be seen, that in the S 1^{+-} and S 1$^+$ stages the basic fraction 1 contained most of the antitreponema activity. In the S 1$^+$ stage, antibodies became detectable in fractions 2 and 3, with fraction 2 containing a slightly higher level of antibody activity than fraction 3. However, in the S II stage, high titers were found in all three

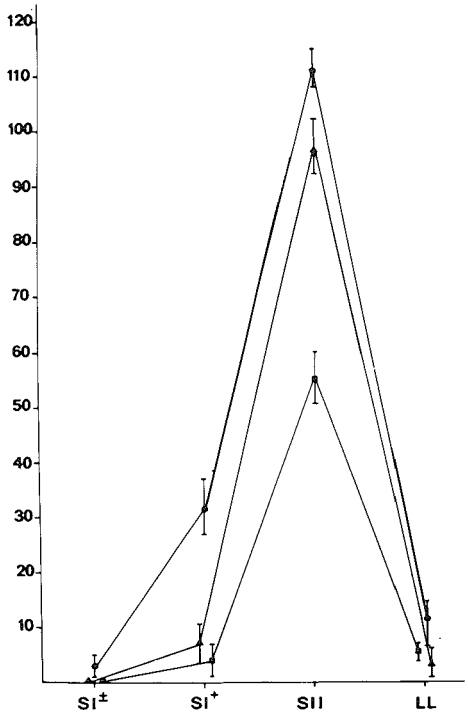

Fig.1. Titration in the FTA-ABS test of IgG1 subclass antitreponema antibodies in three IgG-containing fractions differing in electrophoretic mobility. The initial IgG content was 1 mg/ml.
Symbols: ● , fraction 1
▲ , fraction 2
■ , fraction 3

The stages of human syphilis are shown on the abscissa; titers, as reciprocals of the highest dilutions giving a 1+ positive reaction are shown on the ordinate.

fractions. In fraction 1 the mean titer showed a 3.5-fold increase as compared to the titer of this fraction in the S 1^{+} stage; the increase in fraction 2 and fraction 3 was 14-fold. In the LL stage, the mean titers of fractions 1, 2 and 3 were 1:10.6, 1:3.5 and 1:5.3 respectively. This shows that eventually an even distribution of anti-treponema activity developed in the three electrophoretically different IgG fractions.

Discussion

A comparison of the present results of the FTA- and FTA-ABS tests shows that the demonstration of antibodies of the four IgG subclasses in the S 1^{-} and S 1^{+}_{-} stage patients was different in the two tests. In the S 1^{-} stage, some patients showed positive results for IgG1, IgG2, and IgG3 in the FTA test; in this stage all patients showed negative results in the four IgG subclasses in the FTA-ABS test. In the S 1^{+}_{-} stage, all patients showed positive reactions for IgG1, IgG2, and IgG3 in the FTA test; in the FTA-ABS test, positive reactions were confined to the IgG1 and IgG3 subclasses, with the exception of one patient, which was positive in the IgG2 subclass. These results provide additional support for the specificity of the subclass

antisera. They exclude cross-reactivity between the IgG4 subclass and the other subclasses (FTA test, S 1^{+}_{-} stage), between IgG2 and IgG1 or IgG3 (FTA-ABS test, S 1^{+}_{-} stage) and between IgG1 and IgG2 or IgG3 (FTA-ABS, LL stage). This confirms the reported specificity (15,17,21,31,36) of the subclass antisera from the supplier. Based on this specificity, we conclude that from the end of the primary stage onward, antitreponema antibodies were found in all four IgG subclasses when the FTA test was used and in the IgG1, IgG2 and IgG3 subclasses when the FTA-ABS test was used. These results contradict those of Puritz et al (22) who observed a restriction of antitreponema antibodies to the IgG1 subclass in patients with primary and secondary syphilis.

The complicated structure of the treponemas makes it difficult to compare the results for antitreponema antibodies with the results for subclass antibody formation to better defined antigens. The relatively few published studies of this kind show that, in humans, IgG antibodies to protein antigens (i.e. thyroglobulin (11), diphteria toxoid (25) and tetanus toxoid (31)) are formed in the four IgG subclasses in quantities that parallel the relative amounts of the subclasses in normal serum. With tetanus toxoid there were indications that IgG1 subclass antibodies were produced first (31), but no charge relationship was investigated. However, the nature of the antigen may elicit antibodies belonging to selective subclasses. Antibodies to polysaccharide antigens (8,38) and to grass-pollen antigen during immunotherapy (32) were produced preferentially in the IgG2 and IgG4 subclasses, respectively. It is not known whether such selective mechanisms play a part in the formation of antitreponema antibodies.

The simultaneous appearance of specific antitreponema antibodies of the IgG1 and IgG3 subclasses followed by IgG2 subclass antibodies in syphilis patients resembles the sequence in which the IgG subclasses equilibrate at the basic end after isoelectrofocussing of pooled human IgG (12). IgG1 extends to a higher isoelectric point than IgG3, which in turn extends to a higher isoelectric point than IgG2. Control experiments showed that the absorption procedure efficiently removed antibodies that were reactive with T. phagedenis biotype Reiter from the donor sera as well as high-titered syphilic patient sera. The FTA results demonstrate that the antibody conjugate system used readily delineated the presence of antitreponema antibodies of at least the IgG1, IgG2 and IgG3 subclasses. The present results therefore confirm our previous findings that an antitreponema IgG response starts with basic components of heterogenous IgG. This results in an uneven distribution of

antitreponema IgG antibodies during primary syphilis.

Because IgG1 is present in all heterogeneous IgG, the results with whole sera do not allow for a definite conclusion concerning a shift within the IgG1 subclass. An increase in the IgG1 antibodies could be due to an increase in antibodies already present at early infection or, alternatively, to a spread of IgG1 antibodies to the acidic side of IgG heterogeneity. The titration the IgG fractions of different relative mobilities showed that antibodies are to a large extent confined to the basic fraction 1 during the S $1^{\pm}$ and S 1^{+} stages. In the secondary stage, all three fractions showed high titers, indicating that IgG1 subclass antibodies are subject to a similar shift through IgG heterogeneity. However, during the period between the S 1^{+} and the S II stages a sudden change in the production of IgG1 antitreponema antibodies occurred. In the latter stage high titers of antitreponema antibodies were present in all three fractions. This suggests that as late as the SII stage complete IgG heterogeneity is involved in antitreponema antibody production. This raises the question of whether the extension to more acidic antibodies involves previously uninvolved antigens. This seems likely because several authors (2,9,19,35) showed that with the T.pallidum polypeptides, electrophoretically separated on sodium dodecyl sulphate-polyacrylamide gels, almost all bands react on blots with IgG antibodies in sera from patients with secondary syphilis. However, only a limited number of bands react with IgG antibodies in sera from patients with primary syphilis. The results are similar for IgM antibodies (9,19,35).

This evidence indicates that during primary syphilis, specific IgG antibodies of limited heterogeneity arise against a limited number of treponemal components. These limitations are overcome during the secondary stage. This points to a slow sensitization of the immune system to some of the treponemal antigens. An explanation, which seems the most likely one, is that until the S II stage, an optimally functioning humoral immune system is not stimulated by the relevant antigens. This could be due to the inaccessibility of these antigens, either because they are located inside the treponemas or because they are covered by a protective layer surrounding the treponemas or both. The presence of such a layer was first hypothesized by Christiansen (4). Host serum proteins (1) as well as mucopolysaccharides have been implicated (5,39). Evidence that a layer acts as a barrier inhibiting the reaction of antibodies with treponemal antigens has been obtained from serological tests. No antigen-antibody reactions occurred with freshly harvested treponemas; positive results were obtained only after a period of in

vitro aging (10). This has been attributed to a loss of a protective layer.

A second explanation for the observed limited IgG response is that cellular processes, underlying the production of IgG are suppressed as a consequence of a more general suppression of the cell-mediated immunity during syphilis. There is experimental evidence from in vitro lymphocyte stimulation experiments both supporting and contradicting such a suppression in patients with syphilis. Antigenic stimulation with whole T. pallidum Nichols cells showed a gradually increasing response of lymphocytes isolated from peripheral blood of patients with sequential progressive stages of syphilis (6). This favours the hypothesis of a gradually increasing sensitization to treponemal antigens and contradicts the immunosuppression hypothesis. The evidence favoring suppression stems from the diminished in vitro mitogenic stimulation of peripheral blood lymphocytes from patients. This suppression is maximal during the secondary stage and is attributed to one or more serum factors (16,27). Since we found that the limitations of the initial IgG response are overcome just before the secondary stage, it seems unlikely that IgG production is connected to this postulated immune suppression. This conclusion is supported by the IgG production early in infection, as delineated by the FTA test.

A second observation often cited to support immune suppression during early syphilis is the absence of positive skin reactions to treponemal antigens in patients with primary and secondary syphilis, in contrast to results for patients with later stages of syphilis (14,26). Since IgG production, as well as the elicitation of delayed-type hypersensitivity reactions, requires T lymphocytes presensitized to the relevant antigens, the absence of a positive skin reaction early in infection could be related to the limited sensitization demonstrated here.

The basic IgG response, as delineated by the FTA-ABS procedure, is suggestive of a mechanism involving an inverse charge relationship early in the syphilitic infection (23). Negatively charged molecules, such as mucopolysaccharides, could play a role in this mechanism by modifying the electrical charge of treponemal antigens. In this manner antigens with a net negative carrier component could be provided, which in turn could give rise to the IgG response in the basic region.

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CHAPTER 5.

LIGHT- AND ELECTRON MICROSCOPIC STUDY ON TREPONEMA PALLIDUM (NICHOLS STRAIN) INFECTED RABBIT TESTES, WITH SPECIAL REFERENCE TO THE NATURE OF DEPOSITED MUCOPOLYSACCHARIDES AND LOCALIZATION OF TREPONEMES.

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Accepted by Genitourinary Medicin, 1986.

Summary

The mucopolysaccharide nature of the deposited material in T. pallidum infected rabbit testes was confirmed by histochemical staining with Alcian Blue. Differential staining of mucopolysaccharides revealed the presence of sulphated mucopolysaccharides as an almost consistant feature, while in little more than half of the orchitic testes studied variable deposits of hyaluronic acid were seen. The treponemes were almost exclusively present in the mucopolysaccharide-rich areas. A combination staining with the Warthin-Starry method and Alcian Blue demonstrated treponemes in close association with pre-existing fibrils and cells contained in these fibrils. The latter findings were confirmed by electron microscopy, moreover, the fibroblasts to which treponemes adhered displayed the characteristics of activated cells. The close parallel between the histopathological changes observed here and their descriptions in the literature demonstrates that our specific strain still behaves the same as the original Nichols pathogenic strain of Treponema pallidum.

Introduction

In the course of a syphilitic orchitis in rabbits three sequential but commonly overlapping phases can be distinguished: the inductive, the reactive and the latent phases (1,2). During the inductive phase there is an increase in the numbers of treponemes and the formation of a loosely woven, acellular amorphous substance, which seems to be deposited between the pre-existing testicular tissue. The presence of mucopolysaccharides in these areas was plausible owing to their metachromatic staining with methylene blue and related stains. Scott and Dammin (3) provided evidence that these changes were specific for the syphilitic process. The influx of large numbers of

mononuclear cells marks the transition from the inductive to the reactive phase. The latent stage is characterized by the interstitial fibrosis, which is considered to be a repair mechanism.

Mucopolysaccharides may be of considerable importance in a syphilitic infection, since they have been implicated as a part of a protective cover of the treponemes. Zeigler et al. (4) demonstrated that treponemes in vivo are surrounded by a ruthenium red (RR) stainable substance. This RR positive material most likely consisted of mucopolysaccharides or closely related compounds. In vitro, the co-cultivation of pathogenic treponemes and various types of cultured cells resulted in the adherence of treponemes to these cells (5,6). Some authors consider that this adherence enhances the establishment of infection in a host. Fitzgerald et al. (7) have shown that co-cultivation resulted in cells covered with an amorphous layer of RR stainable material. A similar interaction between the host cells and treponemes in vivo could lead to the production of mucopolysaccharides. This appears to require close contact between treponemes and host cells.

This prompted us to study the nature of the mucopolysaccharides and the localization of the treponemes in relation to host tissue components as a part of the histopathological changes produced by the Nichols pathogenic strain of T. pallidum. This strain has been used in our laboratory for several years.

Materials and Methods

The testes studied were obtained from 70 rabbits which were used in a previous investigation, in which the in vitro survival time of T.pallidum in artificially infected, stored donorblood was determined (8). Orchitis developed in 39 rabbits, in 29 of them the diffuse type of orchitis was observed. The testes of the remaining 10 animals showed the nodular type of orchitis. In 31 animals neither orchitis nor serological evidence of infection was detected. Rabbits with the diffuse type of orchitis were sacrificed when peak orchitis was present. Those with the nodular type were sacrificed at the moment firm nodules could be palpated. Orchitis-negative rabbits were sacrificed after an observation period of 4.5 months and their testes served as controls.

The testes were removed and parts were fixed for light microscopical, histochemical and electron microscopical studies. For light microscopy the testes tissue was fixed in phosphate buffered 4% formaldehyde solution (pH=7.4) and embedded in paramat. (Gurr)

Routine staining of 4 µ thick tissue sections was performed with

hematoxilin- azophloxin.

Mucopolysaccharide specific staining was performed with Alcian Blue 8GX (Aldrich), according to Cook (9) at pH=2.5, using the neutral red modification.

Differential staining of carboxylated and sulphated mucopolysaccharides was done with the High Iron Diamine/Alcian Blue (HIDAB) method according to Spicer (10).

Hyaluronidase treatment of sections was performed with bull testicular hyaluronidase (Hyason, Organon, Oss, The Netherlands). Sections were incubated for one hour at room temperature in a solution, containing 150 I.U. of hyaluronidase in 0.15 M NaCl.

Treponemes were detected using the Warthin-Starry (11) and Steiner (12) staining methods.

A satisfactory visualization of the treponemes in connection with host tissue constituents was produced by the Warthin-Starry method, followed by staining with Alcian Blue as described above.

For electron microscopy small fragments of testes tissue were fixed in a mixture of 4 parts of 40 % formaldehyde and 1 part glutaraldehyde and postfixed with 1 % (w/v) osmiumtetroxide before acetone dehydration. The specimens were embedded in LX 112 (Epon). Semi-thin plastic sections for light microscopy were stained with a freshly prepared 1 % toluidine blue solution in distilled water. Ultrathin sections (computerized LKB ultratome) were mounted on copper grids (300 mesh) and contrasted with uranyl acetate and lead citrate (13). They were examined with a Philips 201 electron microscope.

Results.

Light microscopy.

A prominent histopathological change observed on routine staining of all orchitis positive testes was the occurrence of large, cell-poor oedematous areas in the interstitium of the testicular tissue and also often present in the tunics and paratesticular tissue (fig 1). In these areas a serious destruction of the normal architecture of the testes had occurred. The tubuli seminiferi of the testes showed in all cases a severe atrophy with a reduced spermatogenesis. In some cases there was complete destruction. A second feature was the massive infiltration of mononuclear cells consisting mainly of lymphoid precursor cells and plasma cells. In some of the testes studied, a significant admixture of polymorphonucleated cells was noticed. The oedematous areas were essentially free of the infiltrate which gave the

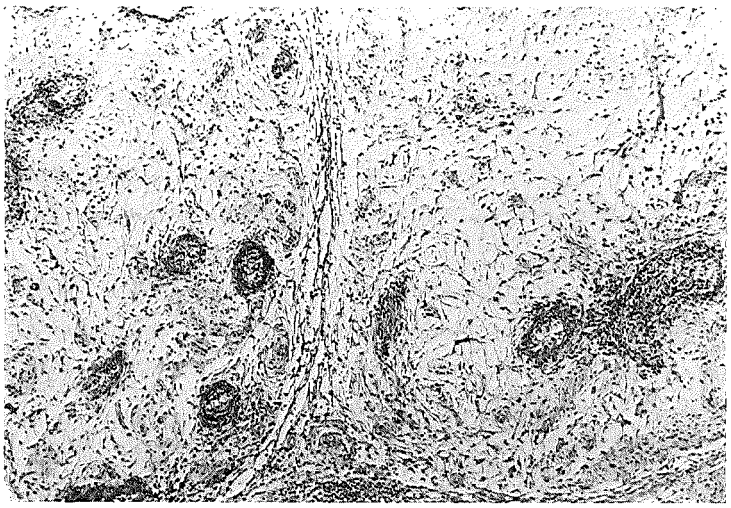
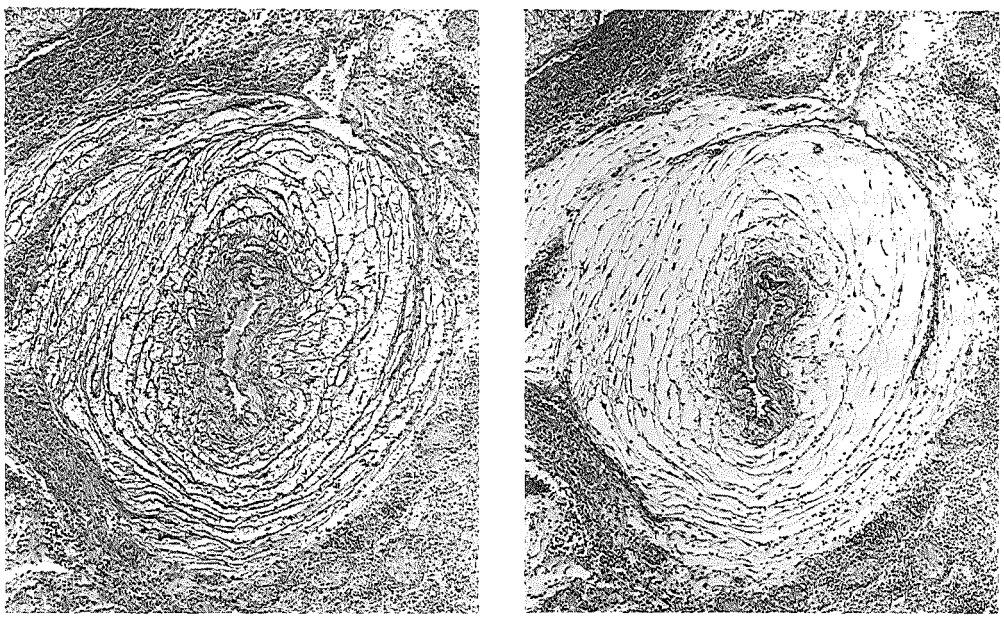


Fig. 1. Section of T. pallidum infected rabbit testis showing optically empty cell-poor areas with vasculitis. (Staining: hematoxilin-azophloxin; magnification 60 x)



A B

Fig. 2. HIDAB stained sequential sections of T. pallidum infected rabbit testis showing a large vessel with a perivascular cell-poor area with an accumulation of mucopolysaccharide-like material. (A) before and (B) after hyaluronidase treatment. (Magnification 60 x)

impression that these areas were surrounded by the infiltrate. Around the blood vessels in these areas the myxoid substance appeared to be arranged in concentric layers. In these vessels a swelling and proliferation of the endothelial cells was observed with occasional thrombus formation in the lumen. A third feature was the interstitial fibrosis which could most easily be observed in regions not obscured by the infiltrate. The blood vessels in these areas showed a prominent vasculitis. In the diffuse orchitis these findings were extended to large parts of the testicular tissue, affecting at least 50% and frequently all the testicular tissue. The same histopathological changes were basically observed in the nodular orchitis and were in restricted areas of otherwise normal looking testes tissue. In a number of the testes with nodular orchitis the aberrations were only seen at the periphery of the testes, in the tunics or/and the paratesticular tissue.

The mucopolysaccharide nature of the mucinous substance was confirmed by Alcian Blue staining of sequential sections from a limited number of testes which were alternately pretreated with or without hyaluronidase. The blue staining, which was present in untreated sections, was completely absent in hyaluronidase treated sections. Similar results were obtained with HIDAB staining of similarly treated sections (fig. 2 A and B).

The differentiation of the deposited material in carboxylated and/or sulphated mucopolysaccharides by HIDAB staining was performed on the testes of the 70 rabbits. Positive staining was only observed in the oedematous areas of testicular tissue of orchitis positive rabbits. In 33 rabbits a black granular staining was seen which appeared to be arranged in fibrillar patterns; in 14 rabbits this was the only staining. It was striking that between these fibrils apparently empty spaces were present (fig 2). In 19 rabbits the black fibrillar staining was accompanied by blue staining areas of various size and staining intensity. In 5 rabbits only a blue staining was observed. In the testes of one orchitis positive rabbit the HIDAB staining failed to demonstrate any mucopolysaccharide. Around the blood vessels the black fibrillar staining was again present in concentric layers. Non-affected parts of these testes did not stain with the HIDAB stain. The testes of the 31 orchitis-negative rabbits were negative when stained with the mucopolysaccharide stains.

Treponemes, as revealed with the Warthin or Steiner staining methods were demonstrated in all testes which showed clinical orchitis. When the Steiner method was used, enormous numbers of treponemes were detected. The impression that these organisms were preferentially present in the oedematous areas,

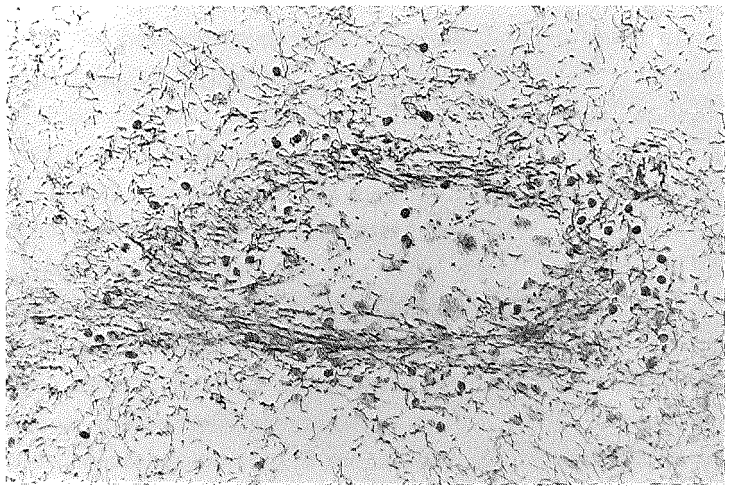


Fig. 3. Section of T. pallidum infected rabbit testis showing enormous numbers of treponemes in a vessel wall and in the mucupolysaccharide-rich areas around this vessel. (Staining: Steiner method, magnification 150 x)

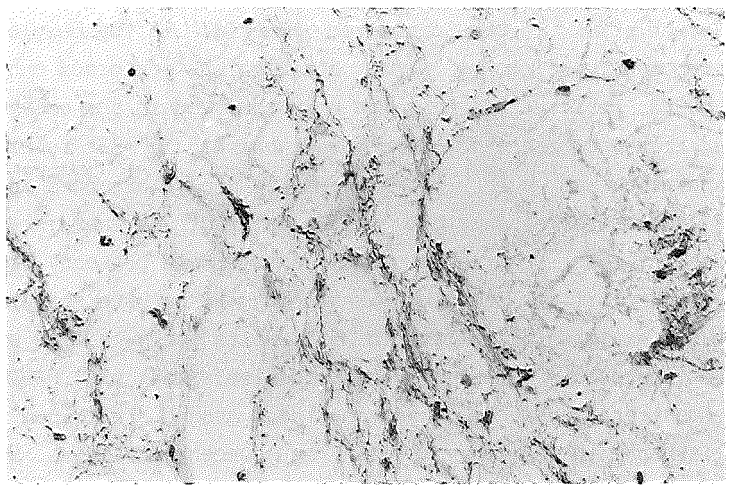


Fig. 4. Low-power magnification demonstrating the non-random distribution of the treponemes in an oedematous tissue area. The treponemes show a preferent localization in close association with tissue components. Note the acellular, amorphous areas between the dark coloured fibrillar structures and the absence of treponemes in these areas. (Staining: Warthin-Starry method, followed by Alcian Blue, magnification 150 x)

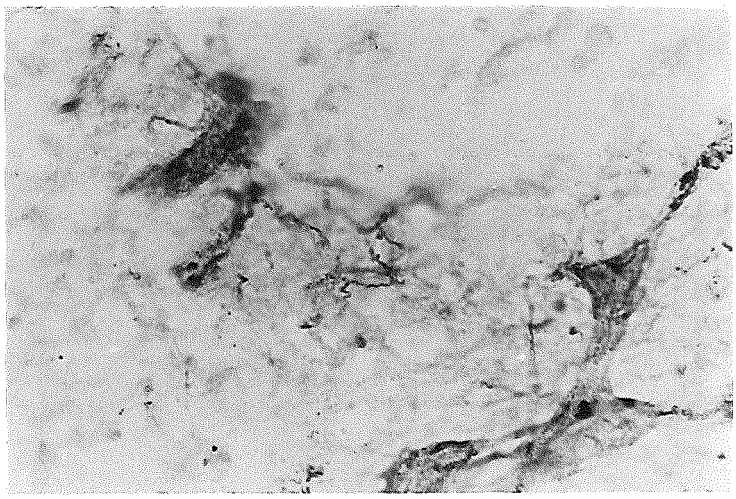


Fig. 5. Larger magnification of the section shown in Fig. 4. It shows the close association between T. pallidum and the tissue cells. (Staining as in Fig. 4. magnification 380 x)

especially around bloodvessels (fig 3) was studied on sequential slides, which were alternately stained with the HIDAB and Warthin stains. The large majority of the treponemes were present in the oedematous areas. Outside these areas each section showed only a few treponemes. The presence of treponemes among the cells of the infiltrate could not be determined with certainty because of its large density. The relationship between the tissue components in the oedematous areas and the treponemes was studied in greater detail using the Warthin method, followed by Alcian Blue staining. Dark-brown coloured treponemes were observed in an apparently close association with fibrillar structures in the oedematous areas. Several treponemes adhering to these fibrils were observed. Moreover, a close association was observed between treponemes and fibroblast-like cells incorporated in these fibrils (figs 4 and 5).

Electron microscopy. The metachromasia of toluidine blue stained semi-thin sections appeared to be very useful in the selection of mesas containing treponemes. The "density" of treponemes in thin sections correlated positively with the presence of metachromasia in semi-thin sections. A considerable number of treponemes were found in the oedematous interstitial spaces of the testes (fig 6), and extracellularly in the walls of the capillary blood vessels. In the oedematous areas the treponemes were in close contact with

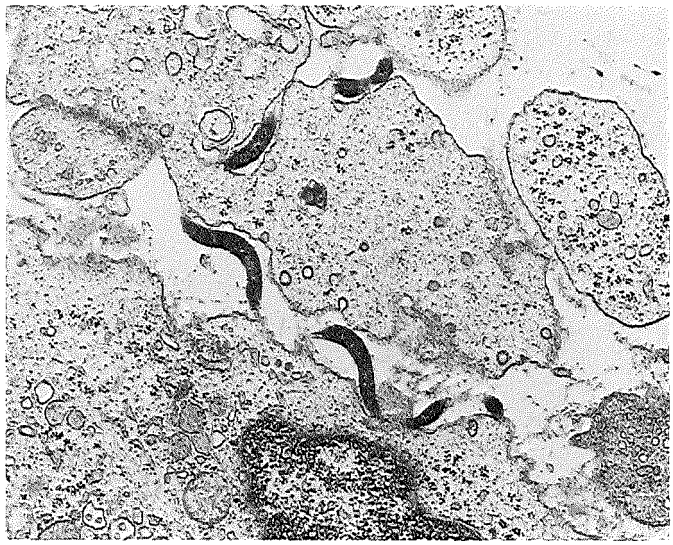


Fig 6. Photo-electron micrograph of an area of T. pallidum infected testicular tissue containing large numbers of treponemes. This area showed metachromasia after toluidin blue staining. (Magnification 30.000 x)

mononuclear cells, mostly fibroblasts. At these places the cell-membrane of the fibroblasts was less visible owing to possible deposition of material. The cells involved were enlarged and showed increased cytoplasmic contents: namely rough endoplasmic reticulum and Golgi apparatus. Less frequently, the treponemes penetrated into cellular invaginations creating gaps without evidence of membrane disruption or fusion of the treponemes with the cellular membrane. Here, the continuity of the cellular membrane was clearly preserved and could be observed electron microscopically (fig 7).

Discussion.

The histopathological changes presently observed on routine staining of infected testes tissue appear to be an admixture of the changes present in the inductive, reactive and latent phases of orchitis (2). These changes have been described to occur simultaneously during orchitis (1). The positive Alcian Blue and HIDAB staining and their inhibition by hyaluronidase are in agreement with earlier findings regarding the mucopolysaccharide nature of the interstitially deposited material (1,3). The Nichols pathogenic strain of T. pallidum, used in the present experiments was obtained in 1975 from Dr J.N.Miller (UCLA,Los Angeles,Cal., USA) and has since then been propagated in

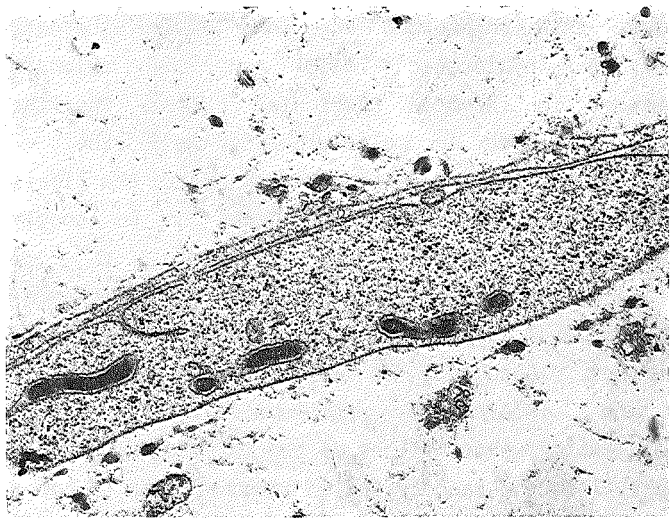


Fig. 7. Photo-electron micrograph of a cytoplasmic protrusion of a fibroblast with treponemes located in invaginations of its cell-membrane. (Magnification 30.000 x)

rabbit testes. The histopathological and histochemical findings demonstrated here are identical to those described in the literature (1,2,3). This demonstrates that our specific strain of T.pallidum still behaves the same as the pathogenic Nichols strain.

The differential staining of mucopolysaccharides with the HIDAB procedure indicated variations in the presence of carboxylated and sulphated mucopolysaccharides. The latter were seen in the large majority of the testes (33 out of 39 orchitis positive testes). A blue staining, indicating the presence of hyaluronic acid was absent in 14 of these rabbits, while in 24 rabbits a staining of variable intensity was observed. We have previously shown that extracts of testes contained increasing amounts of hyaluronic acid with prolonged duration of orchitis (14). Sulphated mucopolysaccharides were undetected in these extracts. This demonstrates that hyaluronic acid can be isolated easily from infected testicular tissue. The variable presence of hyaluronic acid in the testes tissue sections might be related to this easy extractability: hyaluronic acid might have been lost from the tissue sections as a result of the manipulations during the fixation- and staining procedures. The empty spaces between the black fibrils in HIDAB stained sections are highly suggestive of such a loss of material. Moreover, in those sections

showing both types of staining, the blue staining was aligned along the black stained fibrils. This reinforces the idea of an alternative manner of incorporation of hyaluronic acid and the sulphated types of mucopolysaccharides in infected testicular tissue. The fibrillar staining pattern of the sulphated mucopolysaccharides and their resistance to extraction suggest that they are strongly anchored in the fibrillar structures of the testicular tissue. Hyaluronic acid seems to be loosely bound between these fibrils.

There is still no conclusive evidence whether the treponemes or host tissue cells in the presence of treponemes are responsible for the production of mucopolysaccharides. Strugnell et al. (15) have shown that treponemes in vitro do not incorporate radiolabeled precursors into their mucopolysaccharides. However, slices of the infected testes of uni-lateral intratesticularly infected rabbits showed in comparison to the slices from the non-infected, contra-lateral testes, an increased incorporation of radiolabeled mucopolysaccharide precursors ([^{35}S]sulphate and [^{3}H]glucosamine). After penicillin treatment of these rabbits or slices of their testes, the treponemes were effectively killed but incorporation of labeled substances remained higher in the infected than in the non-infected tissue (16). These authors concluded, that the incorporation of radiolabeled precursors represented the biosynthesis of these macromolecules by the host and not by the treponemes and that active syphilitic infection was necessary for the maximum biosynthesis of these macromolecules by the host tissue. Our findings concerning the close association of treponemes with fibroblasts, the activated appearance of the latter and the apparent incorporation of the sulphated mucopolysaccharides into the tissue fibrils support their conclusion. These observations also reinforce the significance of the findings on treponemal adherence to tissue culture cells in vitro. However, the possibility that hyaluronic can be extracted from infected testes only, seems to indicate that hyaluronic acid is produced in excess (14). This could point either to a disturbance of the homeostasis in the production of various types of mucopolysaccharides by the host tissue cells as a result of treponemal infection or to a role of treponemes in the production of hyaluronic acid.

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CHAPTER 6.

MUCOPOLYSACCHARIDES IN SUSPENSIONS OF TREPONEMA PALLIDUM EXTRACTED FROM INFECTED RABBIT TESTES.

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Genitourin. Med. 1985, 61: 7-12

Summary.

The amount and nature of mucopolysaccharides present in extraction fluids routinely obtained in the isolation procedure of Treponema pallidum from infected rabbit testes was investigated. The mean quantity of mucopolysaccharides extracted from both testes of groups of 10 rabbits was 3.09 mg after infection for seven days and 26.88 mg after infection for 12 days, while from the testes of uninfected rabbits a mean of 0.42 mg was obtained. On electrophoresis the isolated mucopolysaccharides showed only one single band with the migration characteristics of hyaluronic acid. This band disappeared completely after pretreatment with hyaluronidase from bovine testes, which showed that during infection with T. pallidum increasing amounts of hyaluronic acid accumulate. They can, at least in part, be extracted by a gentle extraction procedure, suggesting that this material binds loosely. The amount of hyaluronic acid isolated 12 days after infection showed positive correlations with the wet weight of testes as well as the number of treponemes isolated; seven days after infection such correlations were not present.

Introduction.

One of the microscopic features of tissues infected experimentally with Treponema pallidum is the interstitial siting of the infectious process, which has the appearance of loosely woven acellular areas resembling embryonic connective tissue (1,2). In these interstitial areas acid mucopolysaccarides seem to accumulate, as is shown by the presence of a substance which stains metachromatically with toluidine blue and related stains(1,3). The presence of this substance is especially pronounced in rabbits treated with cortisone (4). The metachromasia of such tissues can be abolished by pretreatment with hyaluronidase from bovine testes (1,3). In a chemical analysis of rabbit cutaneous syphilomas Rice found an increase in two types of

mucopolysaccharide: the non-sulphated type was indistinguishable from hyaluronic acid, the sulphated type was identical to chondroitin sulphate C (chondroitin-6-sulphate) (5). In 1963 Christiansen postulated the existence of a mucoid slime layer covering pathogenic treponemes (6), which could explain the presence of pathogenic treponemes in a host side by side with antitreponemal antibodies of high titer. In 1976 Zeigler et al. presented an electron micrographic study of rabbit testicular tissue previously infected with Nichols pathogenic treponemes and of treponemes extracted from such tissue. On staining with ruthenium red, both types of treponemes were seen to be surrounded by a layer of stained material, which probably consisted of mucopolysaccharides or closely related compounds. The extracted treponemes were covered with a less thick and less regular layer of this material than treponemes present in tissue, which indicates that treponemes in vivo seem to be closely associated with the presence of acid mucopolysaccharide. This prompted us to investigate the possible presence and nature of mucopolysaccharides in treponemal suspensions used for in vitro studies.

Materials and Methods.

New Zealand white rabbits about six to eight months old were obtained from a commercial breeding farm. They were housed individually at 18-20 ^{o} C and kept in quarantine for four weeks. The animals were clinically healthy. During the quarantine period the animals were vaccinated against Pasteurella multocida and treated against coccidia with sulphadimethoxine. They were given food free of antibiotics and water ad libidum. All animals used had negative serological reactions for syphilis. It was concluded that they were not suffering from infection with Treponema paraluis cuniculi. T. pallidum (Nichols pathogenic strain) obtained in 1978 from Dr J.N. Miller, UCLA, Los Angeles, USA, was maintained by serial passage in rabbit testes. At weekly intervals, two rabbits were inoculated in both testes with 0.5 ml T. pallidum suspension, containing about 50 x 10^{6} treponemes/ml. One rabbit was killed after seven days, the second after 12 days. The testes were removed and weighed. They were minced with scissors, and 1 ml basal reduced medium (8) containing 20 % fetal calf serum (Flow Laboratories, Irvine, Ayrshire, Scotland) was added for each gram of wet testes. The treponemes were extracted from the testicular tissue by shaking for 45 minutes in an atmosphere of 95 % nitrogen and 5 % carbon dioxide. This extraction procedure was repeated several times as specified in the results section. All fluid extracted was centrifuged at 800 x g for 10 minutes to sediment gross particulate matter. Part of this treponeme

suspension was used for other purposes, the other part was centrifuged at 12.000 x g to remove most of the treponemes. This latter supernate, referred to as testicular extract, was used for estimations of acid mucopolysaccharides.

T. pallidum organisms were counted using microslides of 0.05 mm pathlength (ref.5005 Camlab, Cambridge). The microslide was filled with appropriately diluted treponeme suspension by capillary suction and stuck at both ends to a microscopic slide with nail polish, which also closed the ends of the microslide. The number of treponemes present under the area of a square ocular micrometer had been measured previously using a stage micrometer. The number of treponemes/ml was calculated from the average number of treponemes present in the known volume in the microslide covered by the ocular micrometer. Uninfected testes were obtained from healthy rabbits of comparable age and were processed in the same way as infected testes.

Isolation of mucopolysaccharide. Cetylpyridiniumchloride (CPC) (Sigma Chemical Co, St Louis, USA) was used to precipitate mucopolysaccharide (9). In preliminary experiments optimum conditions of temperature, concentration of CPC, final electrolyte concentration, and relative centrifugal force were assessed. Mucopolysaccharides was isolated as follows: 1 ml of a 1 % (w/v) CPC solution in twice distilled water was added to 2 ml testicular extract, and the mixture was left undisturbed overnight (about 18 hours) at room temperature. The precipitates in the tubes were then collected by centrifugation at 1800 x g for 15 minutes. The supernate was removed very carefully from the loose precipitate, which was suspended in 3 ml precooled (4 ^{o}C) absolute ethanol containing 10 g potassium acetate/litre. After at least 24 hours at 4 ^{o}C the precipitate was collected by centrifuging at 1800 x g for 15 minutes at 4 ^{o}C. The supernate was decanted carefully and the precipitate dried in vacuo. The precipitate was then dissolved in 0.1 ml 2 mol/l sodium hydroxide solution, sometimes with warming in a 37 ^{o}C waterbath, and 1.9 ml bidistilled water was added and the contents of the tubes thoroughly mixed. These solutions were used to measure the mucopolysaccharide content of the testicular extracts. As shown by electrophoresis, these solutions still contained a small amount of glycoproteins precipitated by CPC.

Mucopolysaccharide precipitates free of protein were prepared from testicular extracts as follows: 2 ml extract was mixed with 1 ml 15 % (w/v) trichloroacetic acid in twice distilled water. After four hours at 4 ^{o}C the mixture was centrifuged at 1800 x g for 15 minutes and the precipitate washed three times with 1 ml 5 % trichloroacetic acid solution. Three volumes of

absolute ethanol were added to the combined supernate and washing fluids. After at least 18 hours at 4°C, the gelatinous precipitate was collected by centrifuging at 1800 x g for 15 minutes at 4°C, the supernate removed, and the precipitate dried in vacuo. The precipitate was then dissolved in 1 ml 0.075 mol/l sodium chloride solution. This meant a two fold concentration compared with parent testicular extract. Similar amounts of mucopolysaccharide were obtained from the same testicular extract with both isolation methods.

Calculation of concentration of mucopolysaccharide. The concentration of mucopolysaccharide was calculated from its hexuronic acid content using the borate modification (10) of the carbazol reaction (11). This reaction was performed in a one step modification as described by Pennock (9). Briefly, 0.2 ml of the mucopolysaccharide solution under investigation was mixed in duplicate tubes with 0.2 ml of a solution of 1.25 g carbazol (Merck) in 1 litre absolute ethanol. To this mixture 2 ml borate-sulphuric acid reagent (0.5 g sodium tetraborate (Merck, Darmstadt, West Germany) in 1 litre concentrated sulphuric acid) stored at 4°C was added and thoroughly mixed. The tubes were placed in a vigorously boiling waterbath for at least seven minutes and then cooled. The extinction was read on a spectrophotometer at 530 nm against a blank prepared from the extraction medium by similar treatment as was used for the testicular extract. The concentration of mucopolysaccharide was calculated using a standard curve made with chondroitin sulphate (Sigma, mixed isomers from whale and shark cartilage).

Electrophoresis of mucopolysaccharides. Protein free mucopolysaccharide solutions were subjected to electrophoresis as described by Breen et al. (12) on cellulose acetate strips (Cellogel, Chemetron, Milan, Italy). The electrolyte system for electrophoresis consisted of a mixture of equal parts of 0.05 mol/l lithium chloride and 0.01 mol/l hydrogen chloride solution (pH 2). Samples of the solutions under investigation were applied to the presoaked electrophoresis strips, and current was passed at 4 mA/strip for 25 minutes. The strips were stained with 1 % (w/v) alcian blue 8 GX dissolved in a mixture of equal parts of absolute ethanol and 0.1 mol/l acetate buffer (pH 5.8). Excess stain was removed in a mixture of 5 % (v/v) acetic acid and 10 % (v/v) ethanol in bidistilled water. The strips were clarified in anhydrous methanol for one minute followed by one minute in 20 % acetic acid in methanol. The strips were applied to glass slides and dried. Mucopolysaccharide standards used in the electrophoresis procedure were hyaluronic acid (Sigma, grade IV), chondroitin sulphate (Sigma, mixed isomers, grade III), and sodium heparin, obtained from the University Hospital dispensary. All standards were dissolved

at 1.5 g/l in the extraction medium used to extract rabbit testes. These solutions were processed in the same way as described for the preparation of mucopolysaccharide solutions free of protein. Finally, they were dissolved in 1 ml 0.075 mol/l sodium chloride solution. Serial dilutions of these solutions were used to measure the detection limit of hyaluronic acid and chondroitin sulphate on electrophoresis, which appeared to be 0.15 g/l for both.

Treatment with hyaluronidase. A volume of 0.5 ml mucopolysaccharide solution in 0.075 mol/l sodium chloride was diluted to 2 ml by adding 0.1 mol/l acetate buffer (pH 5). A volume of 1 ml hyaluronidase solution (Sigma, hyaluronidase from bovine testes, grade IV, 810 NV units/mg), containing 0.5 g/l and dissolved in the same acetate buffer, was added and the mixture placed in a 56 °C waterbath for 30 minutes with occasional shaking. The reaction was stopped by the addition of 1.5 ml 15 % trichloroacetic acid solution. The mixture was left undisturbed for four hours at 4 °C and subsequently subjected to the isolation procedure for mucopolysaccharide solutions free of protein as described. Controls were prepared in the same way without the addition of hyaluronidase.

Estimation of protein. Protein was measured by the biuret reaction against the appropriate blanks. Protein concentrations were calculated using bovine serum albumin as a standard.

Statistical analysis. Statistical procedures used were the Wilcoxon non-parametric test for the calculation of significance of differences and the Spearman's correlation test.

Results.

In the first experiments we investigated the yield of mucopolysaccharide in relation to the number of extractions from minced testicular tissue. Seven such extractions were performed on testes obtained from three uninfected rabbits, three rabbits infected for seven days, and three rabbits infected for 12 days. Table I shows the mean amount of carbazol positive material obtained in each extraction from these three groups of rabbits. In uninfected rabbits the amount of mucopolysaccharide obtained was less than 0.05 mg after the first extraction. In the rabbits infected for seven days most of the mucopolysaccharide was obtained from the first two extractions with further extractions yielding a plateau of small amounts. In the rabbits infected for 12 days such a plateau was obtained after the third extraction. The height of these plateaux differed considerably between all groups of rabbits. From Table I it can be seen that the first three extractions yielded 62.5 % of the total

Table I. Mucopolysaccharide obtained from repeated extractions from testicular tissue of uninfected rabbits and those infected with T. pallidum for seven and 12 days (amounts in mg)

Rabbits (3/ group)	Amount obtained from extraction							Total amnt.	Amount(% of total)	
	1	2	3	4	5	6	7		Extr. 1-3	Extr. 4-7
uninf	0.19	0.05	0.01	0.02	0.02	0.04	0.07	0.40	0.25(62.5)	0.15(37.5)
7 d.	1.48	0.46	0.19	0.21	0.24	0.29	0.36	3.23	2.13(65.9)	1.10(34.1)
12 d.	20.00	6.81	3.08	1.09	1.00	1.10	1.09	34.17	29.89(87.5)	4.28(12.5)

extracted from uninfected rabbits, 65.9 % of the total from rabbits infected for seven days, and 87.5 % of the total from rabbits infected for 12 days. In each roup two thirds or more of the total amount was present in the first extraction fluid. From these results we decided to perform three extractions for further work on the isolation of mucopolysaccharide.

Table II shows the results of three repeated extractions from the testes of 10 rabbits in each group. The mean total amount of mucopolysaccharide isolated from the uninfected rabbits was 0.42 mg, from rabbits infected for seven days it was 3.09 mg, and from rabbits infected for 12 days it was 26.88 mg.

Table II. Weight of testes from uninfected rabbits and those infected with T. pallidum for seven and 12 days, number of treponemes obtained from one extraction, and amounts of mucopolysaccharide (MPS) and protein obtained from three extractions of testes (figures are means (SD))

Rab's (10/ group)	Weight of testes	No of trep's ($\times 10^{-7}$)	Amnt of MPS		Amnt. of protein	
			Total (mg)	mg/g testes	Total (mg)	mg/g testes
uninf	5.63(1.21)		0.42(0.15)	0.08(0.03)	97.4(20.0)	16.6(2.0)
7 d.	13.03(2.14)	107.2(51.2)	3.09(2.50)	0.23(0.18)	238.3(55.8)	17.2(3.2)
12 d.	14.65(2.83)	247.0(141.4)	26.88(12.80)	1.79(0.67)	272.8((89.1)	18.2(3.2)

Comparison with uninfected rabbits shows a 7.4-fold increase during the first seven days of infection and 64-fold increase after 12 days- that is, a large increase between days 7 and 12. Table II also shows that the wet weight of testes increased during infection with T. pallidum. During the first seven days the weight of testes increased 2.4-fold ($2\alpha<0.01$), but there was only a slight and not significant further increase between days 7 and 12. When the amount of mucopolysaccharide extracted was related to the wet weight of testes (mg/g) there was a threefold increase in mucopolysaccharide by day 7 and a 22-fold increase by day 12. The mean number of extracted treponemes increased 2.3-fold between days 7 and 12.

The Figure shows that on electrophoresis the extracted mucopolysaccharides consistently showed one single band with the migration characteristics of hyaluronic acid. The extracts obtained from the rabbits infected for seven

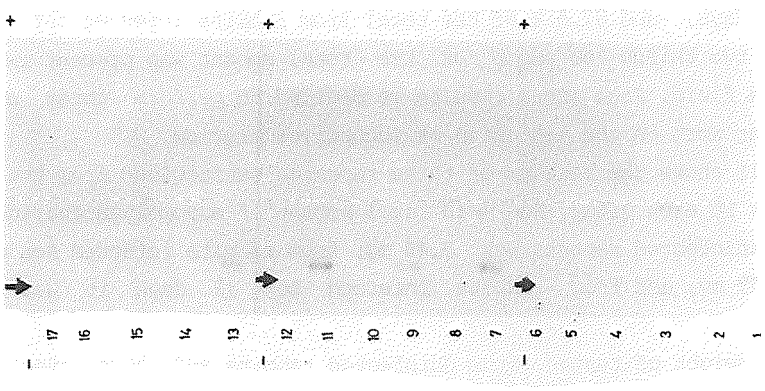

Figure. Cellulose acetate electrophoresis of mucopolysaccharide standards and mucopolysaccharide extracted from uninfected rabbit testes and those infected with T. pallidum for 7 and 12 days, before and after treatment with hyaluronidase, as follows: Hyaluronic acid standard (lane 1); hyaluronic acid standard after treatment with hyaluronidase (lanes 2 and 14); chondroitin sulphate standard (lane 3); sodium-heparin standard (lane 4); protein-free mucopolysaccharide from day 12 infection (lanes 5, 7, 9, and 11); mucopolysaccharide from previous lanes after treatment with hyaluronidase (lanes 6, 8, 10, and 12); hyaluronic acid standard subjected to the hyaluronidase procedure but without hyaluronidase added (lane 13); mucopolysaccharide from unifected testes (no spot visible) (lanes 15 and 16); and mucopolysaccharide from day 7 infection (faint spot visible) Arrows indicate the application lines.

days in most cases contained less mucopolysaccharide than the detectable amount for hyaluronic acid (0.15 g/l). In these cases concentration was necessary to obtain a visible spot. This band also had the migration characteristics of hyaluronic acid. In none of the extracts was a band obtained that corresponded to chondroitin sulphate. This indicates that in the parent extract the concentration of chondroitin sulphate was below 0.075 g/l and presumably much lower, as electrophoresis of concentrated extracts also failed to show chondroitin sulphate. The extracts from uninfected rabbit testes did not yield a visible spot after electrophoresis and staining. In all cases the band disappeared completely after pretreatment of the isolated substance with hyalurondase from bovine testes. Although this type of hyaluronidase splits not only hyaluronic acid but also other types of mucopolysaccharide (13), we conclude from the method of isolation, the reactivity with the carbazol reagent, the electrophoretic mobility, and the digestability with hyaluronidase that the isolated substance was hyaluronic acid.

The amount of hyaluronic acid obtained from rabbits infected for 12 days showed a positive correlation with the wet weight of testes ($r = 0.68$) as well as the number of T. pallidum organisms extracted ($r = 0.87$); these correlations were not present in rabbits infected for seven days.

The amount of protein present in the testicular extract is shown in Table II. The data indicate that protein accumulated in the infected testes during treponemal infection. Although the total amount increased with the duration of infection, the amount of protein per gram of wet testis showed little difference between the three groups of rabbits. The electrophoretic pattern of these proteins, which were present in the extracts prepared in the absence of fetal calf serum, showed the characteristics of rabbit serum proteins as no extra bands were present (data not shown).

Discussion.

We have shown that the extraction of T. pallidum from infected rabbit testes with an aqueous medium yielded a mucopolysaccharide with the characteristics of hyaluronic acid. The amounts isolated from infected testes were well above those obtained from normal testes, while the infected testes yielded an increasing amount the longer the duration of the experimental infection. Sulphated mucopolysaccharides have also been found to be formed during the syphilitic process in rabbit skin (5) and testes (van der Sluis et al., unpublished observation). Using a quantitative destructve technique, Rice

found almost equal amounts (about 20 mg) of hyaluronic acid and chondroitin sulphate per gram of wet syphilitic tissue from rabbits that had not been treated with cortisone. The detection of only hyaluronic acid in the extracts obtained with the mild extraction procedure used here suggests that for different types of mucopolysaccharide different binding types exist within the infected testicular tissue, with loose binding or even simple deposition of hyaluronic acid, while the sulphated mucopolysaccharides seem to be more firmly incorporated into the testicular tissue. Few quantative data are available on the amount of hyaluronic acid present in syphilitic tissue. Millonig, cited by Turner an Hollander (3) isolated one mg hyaluronic acid per gram of wet cutaneous syphiloma tissue from rabbits not treated with cortisone. However, neither the methods of isolation nor of identification of this hyaluronic acid was reported. As already mentioned, Rice found 18.5 mg of mucopolysaccharide like hyaluronic acid per gram of wet tissue (5). Although it is questionable whether cutaneous syphilomas can be compared with infected testes, the amount found by Millonig is confirmed by our results. The data of Rice suggest, however, that it is quite possible that only part of the hyaluronic acid present was isolated by our method.

We showed that the testes increased in weight during the experimental infection with T. pallidum. A similar increase in weight was reported by Baker-Zander and Sell (14). In this present study the increase in weight occurred almost completely during the first seven days of infection; during the following five days there was no further increase in the weight of testes. The oedematous character of syphilitic tissue suggests that a large part of the increase in weight was caused by the increased ability of these tissues to retain fluid. This was reflected in the presence of increasing amounts of rabbit serum proteins in the testicular extracts, which were almost constant in the three groups of rabbits when related to the wet weight of testes. Because of their water binding capacity, the mucopolysaccharides in infected tissue might play a part in the increasing testes weight. The amounts of hyaluronic acid isolated during the infection period studied, however, deviated from the pattern of increasing testes weight. Only moderate amounts were obtained after the first seven days when the largest increase in testes weight was found. During the following five days no further increase in the weight of testes was noted but the amount of extractable mucopolysaccharides increased almost eightfold. Assuming that mucopolysaccharides play a major part in the increase in weight, these results suggest that the production of loosely bound mucopolysaccharide was preceded by the formation of more firmly

bound mucopolysaccharide, which was responsible for the large increase in the weight of testes during the first seven days.

At present it is not clear whether the treponemes produce the mucopolysaccharides or only stimulate their production by host tissue cells. The latter possibility seems feasible as recent work has shown that pathogenic T. pallidum attaches in vitro to many types of cultured human and animal cells (15). It may well be that the newly formed mucopolysaccharides are produced by the fibroblasts as a result of stimulation through treponemal attachment to them. This attachment might also result in incomplete extraction of treponemes, especially during the early stages of the infection. After 12 days of infection, positive correlations were present between the amount of extracted hyaluronic acid on the one hand and the wet weight of testes and the number of extracted treponemes on the other. Incomplete extraction of hyaluronic acid or treponemes, or both, might be the reason that such correlations were not present after seven days of infection.

Our result show that suspensions of T. pallidum derived from rabbits contain hyaluronic acid. As T. pallidum seems to be closely associated with the presence of mucopolysaccharide in vivo, the awareness of the presence of hyaluronic acid in these treponemal suspensions may be of practical importance in in vitro studies.

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CHAPTER 7.

FACTORS THAT INHIBIT THE ADHERENCE OF TREPONEMA PALLIDUM (NICHOLS STRAIN) TO A HUMAN FIBROBLASTIC CELL LINE: DEVELOPMENT IN SERUM OF PATIENTS WITH SYPHILIS

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Genitourinary Medicin 1987, 63: 71-76

Summary.

Serum samples from 25 patients at five different stages of syphilis were investigated for their ability to inhibit the adherence of pathogenic Nichols treponemes to cultured human fibroblasts. Serum taken from patients at the end of the primary stage showed an appreciable inhibition of treponemal adherence, and maximum inhibition of adherence was produced by serum from patients with secondary syphilis. Some freshly harvested treponemal suspensions were resistant to the adherence inhibition factors in serum from patients with syphilis; after incubation in vitro for 24 hours this resistance was lost. In vitro incubation almost doubled the number of adherent treponemes per fibroblast. These phenomena are discussed in terms of loss and reconstruction of the treponemal outer envelope. This leads to the suggestion that adherence occurs not only at the tip of the treponemes, but that surface components are also implicated, possibly as an initial contact mechanism. The composition of the outer envelope may in this way determine localisation versus dissemination of the treponemes.

Introduction

Cocultivation of pathogenic Treponema pallidum and tissue culture cells results in the micro-organisms attaching to the cells (1,2) This adherence prolongs the period of treponemal viability as demonstrated by the retention of motility (1,2) and virulence (3). Pathogenic T.pallidum adhere to a wide variety of human and animal cell types in culture (2). In contrast, non-pathogenic treponemes and killed pathogenic treponemes do not adhere to tissue culture cells (1,2). T.pallidum adhere to the cells by one or both nose pieces. This mode of attachment suggests that the receptors for attachment of

the treponemes are located on their tips (1,2). It is not known to which cell surface components the treponemes attach, but as similar numbers attach to the entire outer surface of each cell, it is believed that these components may be present on the entire surface of all cells. The presence of receptors on pathogenic T. pallidum and their absence from non-pathogenic species has led to the assumption that these structures represent an important factor in the initiation of treponemal infection in the host. Interestingly, a recent study of the successfull in vitro propagation of Nichols pathogenic treponemes used a cocultivaton system of treponemes and tissue culture cells (4). Taken together, these data indicate that the intervention in the attachment of T.pallidum to host cells could prevent the infection. Immune serum from rabbits infected with T.pallidum has been shown to reduce the number of adherent treponemes in vitro (1,2,5). Wong et al. showed that the development of adherence inhibiting factors in infected rabbits took longer than the development of anti-treponemal antibodies (5). Adherence inhibiting factors can be convincingly shown about one month after the immunizing injection with treponemes. No data about the presence and the development of such factors during the course of syphilis in man are yet available. We report here the effect of infected and non-infected human serum on the attachment of Nichols pathogenic treponemes to a human fibroblastic cell line.

Patients, materials and methods

We obtained serum from patients with different stages of syphilis: seronegative, (SI^-), seroconverting (SI^{+-}), seropositive (SI^+), secondary (SII) and late latent (LL). Samples were stored at -70 °C until used. The various stages of the disease occur in the sequence mentioned during the classical course of human syphilis. We used serum from only patients in whom no concomitant disease was diagnosed. The age of patients with early syphilis ranged from 19 to 58 (mean: 34) years and the age of patients in the LL stage ranged between 32 and 72 (mean: 52) years. The case reports of the latter group of patients showed neither any previous history of syphilis nor treatment for syphilis. In all cases their sera gave positive results in the fluorescent treponemal antibody absorbed (FTA-ABS) reaction, whereas the cardiolipin test reactions were negative or only positive at a very low titre.

Control serum samples were obtained from seven healthy blood donors. One of these samples, of bloodgroup AB, was used as a reference serum throughout this study.

A standard set of serum samples was prepared as follows: one sample was

selected randomly from each of the five diagnostic groups of the patients with syphilis. This resulted in a set of five samples that was used in the experiments with all the treponemal suspensions.

Extraction of T. pallidum. T.pallidum (Nichols pathogenic strain) was maintained in male New Zealand white rabbits as described previously (6). The treponemes were harvested from rabbit testes seven and 12 days after inoculation. In all cases orchitis was present. Treponemes were extracted as follows: minced testicular tissue was shaken during 45 min with an equal volume of sterile basal reduced medium (4) containing 20 % heat inactivated fetal calf serum (Flow Laboratories) and 20 mg % (w/v) dithiothreitol. The extraction was performed in an atmosphere of 5 % carbondioxide and 95 % nitrogen. The testicular extracts were centrifuged at 800 x g at room temperature to sediment the particulate matter. Treponemes were counted using microslides as described previously (6). In preliminary experiments the treponemal density and percentage testicular extract seemed to influence the number of treponemes attaching to the cells. In all experiments, therefore, part of the treponemal suspension was centrifuged at 12000 x g for 10 minutes to obtain a supernate free from treponemes. The stock treponemal suspension was adjusted to 1×10^8 using this supernate. This suspension was subsequently diluted 10 times with fresh medium. The working suspension of treponemes thus contained 1×10^7 treponemes per ml and 10% of the particular testicular extract. This suspension was then divided into 1 ml aliquots in small tubes which were loosely plugged with sterile gauze. Some of the tubes were used on the same day (direct experiments) and the others were stored for about 24 hours in a reduced oxygen atmosphere at 33 ^{o}C (delayed experiments). As indicated in the results, fetal calf serum was omitted from the extraction medium in some experiments.

A reduced oxygen atmosphere was established in a dessicator, the final oxygen concentration being 4 %. This was achieved by evacuating the dessicator 4 times to a half atmosphere pressure measured by a manometer. The residual oxygen percentage was measured by a polarographic electrode (type 400, Instrumentation Laboratories) previously calibrated using pure nitrogen, pure oxygen and atmospheric air as reference gases.

Fibroblasts. We used human cultured fibroblasts of cell line TR, which was maintained in glass T-flasks using F 10 medium supplemented with 7% fetal and 7% new-born calf serum (v/v) and penicillin/streptomycin. Confluent fibroblast layers were washed once with phosphate buffered saline (PBS) and the cells were loosened with trypsin (ICN,cat. nr.103140). We then added 5.0 ml medium

and removed the suspended fibroblasts (split ratio 1:2). Plastic petridishes 35 x 10 mm (Costar, nr 3035) equipped with 20 x 20 mm coverglass and each containing 1.5 ml F 10 medium were seeded with a drop of the fibroblast suspension and cultured to 25–50 % confluency at 37°C in an atmosphere of 5% carbon dioxide in air. Before the fibroblasts were used in cocultivation experiments, the medium was removed and the cell layer rinsed once with PBS.

Co-cultivation of fibroblasts and treponemes treated with serum. In the direct and the delayed experiments 0.75 ml of the treponemal working suspension was mixed with 0.25 ml heat inactivated serum and incubated for one hour at 33°C in a reduced oxygen atmosphere. The mixture was then added to the fibroblasts in the petri dishes and incubated at 33°C in reduced oxygen for 2 hours. (Preliminary experiments had shown that maximum numbers of treponemes adhered to the fibroblasts after this time.) The coverslips were then removed from the petri dishes and dipped in PBS to remove unattached treponemes. The coverslips were then laid upside down on the microscopic slides and were sealed with nail-polish. The number of treponemes attached to the fibroblasts were counted by observing by dark field microscopy 50 fibroblasts from duplicate petri dishes. All serum samples were tested in direct and delayed experiments with treponemes obtained from rabbits seven and 12 days after inoculation. The samples from patients from one diagnosis group, together with the standard set of samples, were tested in direct and delayed experiments on two successive days with treponemes obtained from the same rabbit. Each treponemal suspension was described according to the diagnosic group tested. For instance, the treponemal suspension used to test the seropositive (SI^+) serum was designated the SI^+ suspension.

Treponemal motility was measured in wet mounts by observing at least 100 treponemes using dark field microscopy. In all treponemal suspensions treponemal motility was almost 100% directly after extraction from rabbit testes. After overnight incubation in vitro, the motility of the various suspensions ranged from 88 to 96%. At the end of the period of cocultivation with fibroblasts treponemal motility was judged from the treponemes still present in the liquid phase in each petri dish. In the direct experiments, motility was invariably more than 90% whereas in the delayed experiments it ranged from 81 to 90%. This shows that treponemal motility was not selectively affected by the added sera.

Results

To investigate whether serum present in the treponemal suspensions influenced their adherence, we used treponemes extracted in basal reduced medium (BRM) or BRM containing fetal calf serum (FCS). Table I shows that progressively fewer treponemes adhered after adding FCS, AB or S II serum to treponemes in BRM. This shows that under these conditions serum from non-infected people inhibits adherence. When the same serum samples were added

Table 1.

Mean (SD) adherence of T. pallidum (Nichols strain) to cultured human fibroblasts expressed as percentages of control values after adding of 0.25 ml BRM, FCS, AB or LII serum to 0.75 ml T.pallidum suspension, extracted in either BRM or BRM+FCS.

Treponemes suspended in:		Addition of:			
	n	BRM	FCS	AB	LII
BRM	3	100	85.6 (11.7)	74.8 (3.2)	58.4 (5.5)
BRM+FCS	3	100	91.9 (6.0)	93.4 (9.0)	65.0 (2.3)

BRM = basal reduced medium
FCS = fetal calf serum
AB = control serum of bloodgroup AB
SII = serum from patient with secondary syphilis

to treponemes in medium containing FCS, however, only S II serum considerably reduced the number of adhering treponemes. We therefore studied adherence inhibition of serum from patients with syphilis using suspensions extracted in BRM and FCS. This also enabled us to study the effect of the sera after incubation of the treponemes overnight in vitro, as the presence of serum was essential for their survival.

Despite the standardisation of factors, previously shown to influence treponemal adherence (final serum concentration, number of treponemes added and the percentage of testicular extract), the absolute numbers of adherent treponemes/fibroblast varied considerably between the different treponemal suspensions (Table II). This is true for direct and delayed experiments. A second feature shown in Table II was an almost doubling of the number of

adhering treponemes after in vitro incubation. This occurred with seven day as well as 12 day suspensions. The effects of the sera from patients with syphilis were therefore expressed as percentage of the number adherent treponemes/fibroblast after adding standard AB serum.

Table II

Absolute numbers of adherent Treponema pallidum (Nichols strain)/fibroblast in direct or delayed (24 hours later) experiments with treponemes harvested seven or 12 days after inoculation in rabbits and extracted in BRM+FCS medium with 0.25 ml AB serum added/ml suspension.

Treponeme suspension	7-days		12-days	
	dir.	delay.	dir.	delay.
SI^{-}_{-}	9.2	19.9	7.1	15.9
SI^{+}_{-}	10.9	19.2	11.3	21.6
SI^{+}_{+}	9.8	19.5	18.5	26.6
SII	17.2	27.8	12.8	19.8
LL	13.7	24.6	8.4	16.5
M (SD)	12.2(3.3)	22.2(3.8)	11.6(4.5)	20.1(4.3)

Figure 1 shows the effects of serum samples from the control and the five diagnostic groups. It can be seen, that the control sera neither promoted nor inhibited the attachment of treponemes compared to the standard AB serum. In the delayed experiments the addition of sera from patients at some stages of syphilis resulted in partial inhibition of treponemal adherence with seven and 12 days suspensions. With both seven and 12 day suspensions, the adherence of the treponemes was considerably reduced by serum from seropositive patients. The number of adhering treponemes differed from that adhering with control serum by more than one standard deviation. Maximum adherence inhibition was obtained by serum from patients with secondary syphilis in delayed experiments with seven day (44% of adherence inhibited) and 12 day (57% of adherence inhibited) suspensions. Serum from seroconverting patients exerted an intermediate effect; in the delayed experiments with 12 day suspensions the adherence differed one standard deviation from that of the control sera, whereas in the delayed experiments with seven day suspensions the degree of adherence overlapped with that of the control sera. Serum from patients with

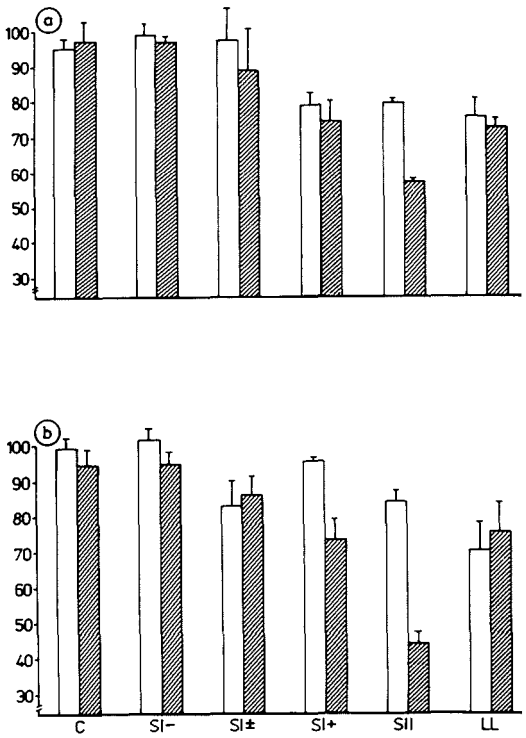

Fig 1. Inhibition of adherence of T. pallidum (Nichols strain) to human fibroblasts by serum from patients with five different stages syphilis (S 1^- = seronegative, S $1^{\pm}$= seroconverting, S 1^+ = seropositive, SII= secondary and LL= late latent) and healthy controls (C) using treponemes harvested from rabbits seven days(a) and 12 days(b) after inoculation. Open bars: direct; hatched bars: delayed experiments. Ordinate: percentage of number of treponemes/fibroblast with standard AB serum added.

late latent syphilis inhibited adherence by about 25% with both seven and 12 day suspensions. As the motility of the treponemes at the end of the cocultivation period was between 81% and 90% in the delayed experiments, the reduced adherence was unlikely to have been caused by differences in treponemal motility due to incubation with different sera.

In the direct experiments, less clear cut results were obtained. With seven days suspensions the adherence inhibition produced by serum from patients with secondary syphilis was the same as that produced by serum from seropositive patients and those with late latent latent syphilis. In direct experiments with 12 day suspensions using the serum from patients with seropositive patients and those with secondary syphilis, a resistance to adherence inhibition was seen. As shown in fig. 1, these sera produced a definite adherence inhibition with the same treponemal suspensions that had been incubated overnight.

As the inhibitory activity of sera of patients with syphilis was analysed by diagnostic group using one isolate of treponemes, it could not be shown whether this resistance was associated with the syphilitic sera or with the

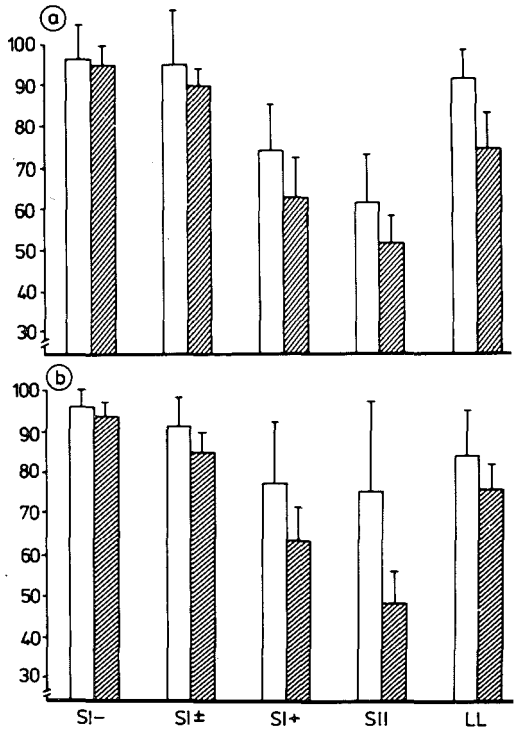

Fig 2. Mean inhibition of adherence of T. pallidum (Nichols strain) to human fibroblasts by standard set of serum samples using 5 different treponemal suspensions with treponemes harvested from rabbits seven days (a) and 12 days (b) after inoculation. See fig 1 for abbreviations.

treponemal suspensions. The set of standard sera was therefore included in each experiment. Fig 2 shows the mean effect of these standard sera on the five seven day and five 12 day treponemal suspensions. In the experiments using seven day suspensions these standard sera produced a similar profile of adherence inhibition as was shown in fig 1(a) for the delayed experiments that used seven day suspensions. With the directly used 12 day suspensions (fig 2b) the mean effect of standard sera of seropositive patients and those with secondary syphilis was only a modest reduction in the treponemal attachment. From the large standard deviation, however, it was clear that there was a wide variation in susceptibility to the adherence inhibition factors among these treponemal suspensions. The 12 day suspensions were susceptible to these factors in delayed experiments, and the standard deviation had diminished drastically. Fig 3 shows a typical example of the addition of the standard sera to a resistant treponemal suspension. It can be seen that such suspensions were, on the day of their isolation, almost completely insensitive to the adherence inhibitory factors in the whole set of standard sera.

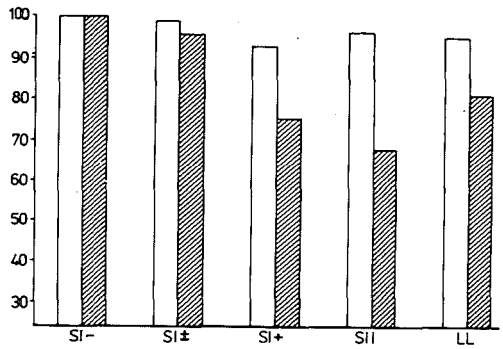

Fig 3 Inhibition of adherence of T. pallidum (Nichols strain) to human fibroblasts by set of standard serum samples using treponemes harvested from a rabbit 12 days after inoculation. See fig 1 for abbreviations

Discussion

The results presented show that the addition to treponemal suspensions of control serum as well as that from patients with syphilis reduces the attachment of treponemes to cultured human fibroblasts. This indicates that the serum samples contain factors that interfere with the adherence process. The serum samples from patients with syphilis exerted a stronger effect than those from the controls. This was especially apparent in the delayed experiments. Using serum samples from the sequential stages of classic syphilis in man, the ability of the syphilitic serum to inhibit adherence was first found to exceed that of the control sera at the end of the primary stage. It reached its maximum during the secondary stage and was still detectable during the late latent stage. This profile of development of the adherence inhibition factors in these serum samples resembles that of serum immunoglobulin concentrations (7,8), anti-treponemal antibody titers (8) and the number of treponemes assumed to be present during the course of the infection.

Immune sera from syphilitic rabbits have been shown to contain adherence inhibition factors (1,2,5). Wong et al studied the development of these factors during the course of experimental syphilis (5). They showed that serum did not inhibit treponeme adherence until about one month after rabbits were inoculated, which was well after serological reactions, including those to the T. pallidum immobilisation (TPI) test, became positive. In the present study, the adherence inhibition factors were at first shown during the seropositive stage. This stage is defined as the first stage of primary syphilis in which the four serological tests, including the TPI test, give positive results (9).

to parallel the development of the serological reactions more closely.

In the direct experiments the demonstration of the adherence inhibition factors was more ambiguous. Some of the suspensions used were resistent to the adherence inhibiting action of serum of patients with syphilis. Our results strongly suggest that this resistance was associated with the treponemal suspensions themselves, rather than with the added sera, especially as this resistance was lost after incubation overnight. One explanation could be that these particular suspensions contained substances of either testicular or treponemal origin, which inactivated the adherence inhibiting components of the serum from patients with syphilis. In that case, the apparent absence of such factors after incubating the treponemal suspensions overnight in vitro could be due to the labile nature of the factors or because they were inactivated by the treponemes during incubation. A second explanation, which seems to be more plausible, is that the change in susceptibility to adherence inhibition factors after in vitro incubation was associated with changes in the treponemal surface. Freshly harvested treponemes have been shown to be covered with host proteins (10) and with a slime layer probably consisting of mucopolysaccharides (11). Gradual dissipation of these substances from the treponemal surface during in vitro incubation could unmask treponemal surface components that act as adherence sites. The inability to detect reactions between antigen and antibody in certain serological tests that use freshly harvested treponemes has also been ascribed to the masking of treponemal components by an extracellular protective layer (12). The present observation that the absolute numbers of adhering treponemes increased appreciably after overnight incubation may also be explained in terms of exposure of the binding sites on the treponemal surface.

Adherence inhibition by serum from patients with syphilis could be explained by reoccupation of exposed treponemal sites by constituents from the added serum. Several authors have reported that the effect of syphilitic rabbit serum on the treponemal adherence favour such a role for specific antibodies in the adherence inhibition (1,2,5). The results with whole serum, that was used in these studies, however, do not provide conclusive evidence that antibodies are the sole cause of adherence inhibition. Occupation of the exposed treponemal surface structures by serum components other than antibodies could probably also reduce the tendency of the treponemes to adhere to cultured cells. Alderette and Baseman identified several host serum proteins that are able to combine with the outer surface of the treponemes (10). Peterson et al showed that washed T. pallidum avidly bind fibronectin

to their surface (13). These authors also demonstrated that this interaction used three outer envelope proteins of T. pallidum, which had previously identified as possible receptor proteins in the adherence of T. pallidum to cultured cells (11). Recently, Fitzgerald and Repesh provided evidence that host fibronectin was present not only at the tips, but also on the entire outer surface of Nichols treponemes adhering to cells (15). Combination of these proteins with the exposed treponemal outer surface components could lead to the re-formation of a protective layer, which in turn could lead to a diminished treponemal adherence. This would implicate that the whole outer surface of treponemes is involved in the treponemal adherence. Electron microscopic observations, however, have shown that treponemes adhere to cells by means of their tips (1), which suggests that exposed outer surface components of the treponemes might function as a first contact mechanism between treponemes and cultured cells.

The wide variation in the number of adhering treponemes per fibroblast using different treponeme suspensions suggests that the treponemes used in the present study differed in the composition of their extracellular layer. Suspensions, resistant to the adherence inhibition factors of serum from patients with syphilis showed, in the direct experiments, an absolute number of adhering treponemes which was well above the mean of that particular group. This suggests that exposure of those treponemal components detected by serum from patients with syphilis is not an indispensable condition for adherence to occur. The adherence phenomenon may be divided in two processes, however, the first one being independent of the exposure of the sites influenced by serum from patients with syphilis and the second depending on the exposure of such sites. One could speculate whether the composition of the extracellular layer determines the selective localization of the treponemes in areas rich in connective tissue or the dissemination of the treponemes.

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ADDENDUM
TO
CHAPTER 7

In this addendum the investigations of some aspects of the adherence of the treponemes to cultured cells and their possible interaction with serum proteins are described. Unless stated otherwise the materials and methods used are the same as in Chapter 7. All sera and serum fractions that were used were heat-inactivated.

The literature findings concerning the adherence indicated that viable, motile treponemes adhered to tissue culture cells, but that non-motile treponemes failed to do so. We investigated these findings using freshly isolated treponemes (referred to as fresh treponemes) and treponemes that had survived in vitro for 24 h (aged treponemes) and their respective counterparts that were killed by heat. The treponemes were killed by incubating them for 2 h at 56^{o} C. Darkfield examination showed that no motile treponemes were left after this treatment. The adherence of the following five varieties of four different isolates were studied. These were a. viable fresh, b. killed fresh, c. fresh treponemes that were killed on the day of their isolation and which had been stored for 24 h, d. viable aged treponemes and e. killed aged treponemes. The results are depicted in Table 1. It can be seen that killed fresh treponemes did not adhere to fibroblasts. This is in contrast to the adherence that is demonstrated by their viable counterparts. The viable aged treponemes adhered in larger numbers to the cells than did the viable fresh treponemes. However, the aged killed treponemes also adhered in considerable numbers to the cells. Since the killed fresh treponemes, that had been stored for 24 h did not adhere, the adherence of killed aged treponemes could not have been due to storage. Therefore, these results suggested that during the storage of the viable treponemes changes occurred in their outer surface. These changes were dependent on the viability of the treponemes and were therefore the result of an active process. On the other hand the adherence displayed by the killed aged treponemes was not dependent on the viability. This showed that the adherence of the aged treponemes may be a passive process. This could have increased the adherence capacity of the viable aged treponemes for fibroblasts.

In Chapter 7 it was discussed that the adherence inhibition of the treponemes to the fibroblasts by syphilitic sera might be related to the

Table 1.

Mean numbers (SD) of adherent treponemes/fibroblast after various pre-treatments as indicated. The results of four different isolates are shown.

Fresh treps	Aged treps	Killed fresh treps	Killed fresh treps, 24h	Killed aged treps
15.9 (2.6)	25.5 (5.3)	0.6 (0.3)	0.3 (0.2)	11.8 (4.5)

capacity of the treponemal outer membrane to interact with several serum proteins. Among these are antibodies directed against the treponemes. Furthermore, fibronectin, which is considered to be the major ligand of the cells in the adherence process, is also present in serum. An attempt was made to characterize the adherence inhibition factors which were present in one of more of the fractions which were obtained by Sephadex G-200 column chromatography of the syphilitic sera. The three fractions obtained (referred to as fraction 1, fraction 2 and fraction 3, according to the sequence in which they were eluted from the column) from each of the three sero-positive primary ($S\ 1^{+}$) and the three secondary (S II) syphilitic sera were concentrated to the volume of the serum from which they were derived. Next, they were dialyzed against 100 volumes of basal reduced medium. Their capacity to inhibit the adherence of the treponemes to the fibroblasts was compared to the inhibition effect of the corresponding fractions from a standard control serum. It can be seen in Table 2 that the whole $S\ 1^{+}$ and S II sera and their respective fractions 1 weakly inhibited the adherence of the treponemes when 7 day fresh treponemes were used. When 12 day fresh treponemes were used, the sera and their respective fractions 1 produced a weak, if any, adherence inhibition. This might be related to the refractory character of the 12 day fresh treponemes to the adherence inhibition factors that was previously observed (see Chapter 7). However, when aged treponemes were used, the sera and their respective fractions 1 inhibited the adherence, irrespective of whether the treponemes were 7 days or 12 days. Fractions 2 and 3 from the sera of patients with sero-positive primary syphilis did not reduce the adherence of either the fresh or the aged treponemes. This is also true for the fractions 2 and 3 from the sera of patients with secondary syphilis,

Table 2.

Mean percentage (SD) of adherence of fresh and aged treponemes after pre-incubation with three sero-positive primary ($S\ I^+$) and three secondary syphilitic (S II) sera and their respective fractions which were obtained upon Sephadex G-200 gel filtration.

Day	Type Treps	Serum Type	whole	fraction 1	fraction 2	fraction 3
7	fresh	$S\ 1^+$	89.5(4.6)	84.7(10.2)	95.8(16.9)	97.7(11.8)
	aged		68.8(17.5)	70.2(17.7)	97.8(5.0)	96.2(4.5)
12	fresh	$S\ 1^+$	95.4(13.0)	93.4(7.4)	100.7(4.4)	99.4(1.7)
	aged		75.1(15.1)	83.6(14.5)	100.9(0.5)	101.6(6.0)
7	fresh	S II	83.1(11.5)	84.1(5.5)	96.1(15.0)	96.4(7.3)
	aged		59.6(12.3)	62.9(17.0)	86.5(11.2)	96.3(2.2)
12	fresh	S II	92.1(8.4)	88.8(13.8)	101.3(2.8)	101.3(2.6)
	aged		55.9(9.4)	68.8(7.3)	92.5(9.4)	99.9(8.2)

when used with fresh treponemes. However, a limited inhibition of the adherence was produced by the respective fraction 2 of the S II sera when they were used with aged treponemes. The fractions 2 contains the antibodies, except those of the IgM class. These findings suggested a role for anti-treponemal antibodies in the adherence inhibition. Since a similar adherence inhibition of aged treponemes was not observed using the fraction 2 from the $S\ 1^+$ sera, this suggests and is compatible with a role of antibodies of the IgG class. This type of antibodies has been shown to develop slowly during early syphilis. The majority of the factors that inhibited the adherence of the treponemes was contained in fraction 1 of the syphilitic sera. Since this fraction contains the macromolecular species of the serum proteins, the presence of the adherence inhibition factors in this fraction is compatible with the molecular mass of fibronectin (450 kD) as well as with the presence of IgM (900 kD). Both these proteins might be involved in the adherence inhibition of the treponemes. An attempt was made to differentiate

between both these proteins by absorbing fraction 1 with gelatin-sepharose. The efficacy and specificity of this absorption procedure for the isolation of fibronectin from plasma has been reported (1). The absorption of the various fractions was performed on small columns of gelatin-sepharose (Pharmacia) prepared in syringes. The absorbed material was expelled by centrifugation. Controls were prepared by treating the fractions 1 with sepharose alone (Pharmacia) in a similar manner. The IgM contents of the absorbed fractions was measured by the Mancini method and expressed as a percentage of the IgM contents of the untreated respective fractions. The treponemal adherence inhibition capacity of the absorbed and unabsorbed fractions 1 was expressed as a percentage of the adherence observed when the untreated fraction 1 prepared from the control serum was used. The results are shown in Table 3. It can be seen in Table 3 that fraction 1 contained adherence inhibition factors, that inhibited aged treponemes to a greater extent than fresh treponemes. The absorption with gelatin-sepharose increased the adherence of the fresh treponemes to a level that was only slightly below the level that was obtained when fraction 1 from the control serum was used. However, when aged treponemes were used, the absorption with gelatin-sepharose of the fractions 1 from the sera of patients with sero-positive primary syphilis produced a reduction of about 25 % in adherence inhibition of

Table 3.

Mean percentage of adherence (SD) of treponemes that had been pre-incubated with the unabsorbed, gelatin-sepharose and sepharose absorbed fractions 1 that were prepared by Sephadex G-200 gel filtration of serum[a]. Relative IgM content of these fractions is also shown.

Sera	Treps	Fr.1 untreated	Fr.1 gel.seph.	Fr.1 seph.	IgM Fr.1 gel.seph.	IgM Fr.1 seph.
Sl^+	fresh	81.2(5.8)	95.9(4.4)	80.1(5.2)	99.3(3.2)	95.4(4.8)
	aged	66.0(7.9)	90.6(5.7)	66.4(8.3)		
SII	fresh	76.3(9.3)	95.2(5.3)	80.5(7.6)	92.3(3.0)	92.3(3.0)
	aged	53.3(1.1)	80.1(5.6)	56.5(9.8)		

[a] The same sera as shown in Table 2 were used.

treponemes, but the level of adherence that was obtained in the presence of the fraction 1 from the control serum was not reached. The same was true for the gelatin-sepharose absorbed fractions 1 from the sera of patients with secondary syphilis. The controls were prepared by absorbing the fractions 1 with sepharose and contained treponemal adherence inhibition capacity which was identical to that present in their unabsorbed counterparts. The two different manners of absorption of the fractions 1 showed no differences in the relative IgM contents of the absorbed fractions. These results indicated that fibronectin was presumably a major adherence inhibition factor that was present in fraction 1. However, using aged treponemes a residual capacity of adherence inhibition was observed in the gelatin-sepharose absorbed fraction 1. This was indicative of a role of the IgM class antibodies in the adherence inhibition. The fact that this was observed only when aged treponemes were used is compatible with the observations that antibodies can only interact with treponemes after in vitro aging.

Table 4.

Mean percentage (SD) adherence of treponemes that were pre-incubated with the sera of four patients with secondary syphilis, that were preabsorbed with fresh and aged treponemes.

	fresh treponemes			aged treponemes		
absorbence	none	fresh treps	aged treps	none	fresh treps	aged treps
	81.6 (7.4)	100.9 (11.8)	113.1 (15.2)	45.8 (9.1)	48.8 (12.4)	47.6 (13.3)

Finally, the question whether the adherence inhibition factors could be removed by the absorption of the syphilitic sera with treponemes was investigated. Since differences seemed to exist between fresh and aged treponemes with respect to their sensitivity to such factors, four sera from patients with secondary syphilis were absorbed using both types of treponemes. The absorptions were performed by resuspending a pellet containing 3×10^7 treponemes in one ml of heat-inactivated syphilitic serum. After an

incubation for one h at 33° C in a reduced oxygen atmosphere, the treponemes were removed by centrifugation for 10 min at 12,000 x g. The absorbed sera were stored at -70° C until use. The motility of the aged treponemes that were used for the absorptions was >90 %. The results shown in Table 4 are expressed as a percentage of the adherence of the treponemes that was observed in the presence of the control serum. It can be seen in Table 4 that when the preabsorbed sera were used with fresh treponemes, the adherence inhibition factors of the sera were removed by the fresh as well as the aged treponemes. A higher percentage of treponemes adhered to the fibroblast after the absorption of the sera with the aged treponemes. However, when the aged treponemes were pre-incubated with the absorbed sera, the percentages of the adherent treponemes did not change as compared to the incubation with the control serum.

In summary, we have shown that in vitro storage produced changes in the outer surface of treponemes. These changes were demonstrated by the capacity of the aged killed treponemes to adhere as compared to the fresh killed treponemes which did not adhere. Since fresh killed treponemes that had been stored, did not adhere, the occurrence of these changes was dependent on the viability of the treponemes. Moreover, these findings indicated that the adherence of the fresh viable treponemes was an active process, whereas the adherence of the aged treponemes could be partially a passive process.

The studies with the fractionated syphilitic sera indicated that for fresh treponemes, fibronectin was most likely the major adherence inhibition factor present in the syphilitic sera. However, for the aged treponemes there was evidence that, in addition to fibronectin, anti-treponemal antibodies took part in the adherence inhibition. A role for IgM was demonstrated in fractions 1 which were prepared by Sephadex G 200 gelfiltration of the sera of patients with sero-positive primary and secondary syphilis. The possible participation of the antibodies of the other immunoglobulin classes was observed in the gelatin-sepharose absorbed fractions 2 which were obtained by Sephadex G-200 fractionation of sera from the patients with secondary syphilis. The participation of these antibodies during the secondary stage and not during the sero-positive stage is compatible with a slow development of the specific IgG response.

The absorption of the sera from patients with secondary syphilis with fresh or aged treponemes produced a reduction in the capacity of these sera to inhibit the adherence of fresh treponemes. However, no effect was observed when aged treponemes were used with the absorbed sera. These differences

might be explained by the assumption that the factors to which aged treponemes are vulnerable were not completely absorbed out by the relatively low numbers of treponemes that were used. The fibronectin that was demonstrated to be successfully removed by the use of fresh treponemes, can be replaced by other adherence inhibition factors, most likely antibodies. The fact that this could only be demonstrated with aged treponemes is compatible with the changes occurring in the treponemal surface upon in vitro aging and with the observation that only aged treponemes are accessible to antibodies. However, it is not known whether these antibodies include antibodies against the fibronectin-binding site of the treponemes.

Literature.

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CHAPTER 8.

THE INACCESSABILITY OF ANTI-TREPONEMAL ANTIBODIES TO THE OUTER MEMBRANE OF ADHERENT TREPONEMA PALLIDUM (NICHOLS STRAIN)

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Submitted for publication.

Summary

Fresh and aged adherent T. pallidum, were used to study the accessability of their outer membrane to antibodies by means of an indirect immunofluorescent technique. The integrity of the outer membrane was demonstrated by the non-reactivity with a monoclonal antibody directed against the axial filaments. IgG and IgM antibody fractions from the sera of patients with secondary syphilis, gave with the fresh fibroblast-adhering treponemes a mean of 14.5 % IgG- and of 43.2 % IgM positive treponemes. These means were 32.1 % and 87.3 % respectively for aged treponemes. Lower percentages were observed when fibronectin adhering treponemes were used. This demonstrates the inertness of the outer membrane in the binding of antibodies in a majority of the fresh treponemes, which is partly lost on in vitro aging. Using the sera from patients with sero-positive primary and secondary syphilis no binding of IgG and IgM antibodies was observed. This was also observed using sequential incubations with the antibody fractions and control sera. This was accompanied by the deposition of the third complement factor (C3) around the treponemes. Incubations of IgG- or IgM pre-coated adherent treponemes with heat-inactivated control sera, a C3 deficient serum or a Clq deficient serum did not result in the deposition of C3, but only partially restored the detection of human antibodies. The absence of C3 after the incubation with the Clq deficient serum excludes a spontaneous absorption of C3 as well as an activation via the alternative pathway. The deposition of C3 from control sera most probably points to the classical pathway of complement activation and suggests that antibodies constitute a part of the protective cover of treponemes.

Introduction

One of the intriguing aspects of syphilis is that during the disease anti-treponema antibodies are produced, but, which do not lead to a rapid eradication of the treponemes. Although these antibodies may modify the course of the infection, they do not provide protection in such a manner as to prevent the disease from reaching latent infection and eventually the tertiary stage in some cases. Recent investigations, using the highly sensitive Western blotting and immunoprecipitation techniques have shown that almost all polypeptide bands obtained from solubilized Treponema pallidum react with IgG and IgM antibodies from sera of patients with secondary syphilis (3,12,14,18) or rabbit immune sera (11). These preparations most probably contain polypeptides that are derived from all treponemal structures. However, as discussed by Stamm and Bassford (17), the proteins that are situated on the treponemal cell surface are especially of interest for several reasons. Among them is the notion that a protective host response presumably must be targeted against antigenic determinants exposed on the cell surface of the treponemes. Surface radiolabeling of viable treponemes revealed the presence of several immunogenic polypeptides, which could be precipitated by antibodies from rabbit immune sera (1) as well as sera from patients with secondary syphilis (15). One way in which the treponemes may manage to escape destruction in vivo is by covering themselves with an extra-cellular layer, which renders their outer membrane inaccessible to the antibodies. A hypothesis dealing with such a layer was formulated by Christiansen (7). Mucopolysaccharides (7,9,23) and host serum proteins (2,13) have been implicated as constituents of a protective cover. However, as yet, it has to be proven that the substances present on the treponemal surface act as a barrier in preventing the access of antibodies to the antigens of the outer membrane.

In the present experiments the treponemes were attached to cultured fibroblasts or fibronectin by their spontaneous adherence to these substrates. In this manner native treponemes could be used without a fixation procedure, which is used in the FTA-ABS test and which could possibly introduce changes in the structure of the treponemes. The adherent treponemes were used as substrates in indirect immunofluorescence studies to investigate the accessability of anti-treponemal antibodies to the treponemal surface antigens.

Materials and Methods.

Treponema pallidum, Nichols pathogenic strain, was maintained in New

Zealand white rabbits, as described previously (21). The rabbits were killed on day 7 and day 12 after inoculation with approximately 2.5×10^7 treponemes per testis. Orchitis was present in all cases. Unless stated otherwise, 1.0 ml of basal reduced medium (BRM) (8), containing 20 % (v/v) fetal calf serum (FCS) (Flow Laboratories, Irvine, Ayrshire, Scotland) was added per gram (wet) minced testicular tissue and treponemes were extracted by shaking during 45 min. in an atmosphere of 95 % nitrogen and 5 % carbondioxide. The treponemal density was adjusted to 1×10^8 treponemes per ml by addition of a treponeme-free supernatant and subsequently to 1×10^7/ml by dilution with fresh BRM-FCS. The treponeme-free supernatant was prepared by centrifuging a part of the original suspension at 12,000 x g for 15 min. The treponemal suspension was divided in aliquots of 1 ml. A part was used on the same day (fresh treponemes), the other part was stored under a reduced oxygen atmosphere (20) and was used the next day (aged treponemes). The motility of the aged treponemes, as determined by observing at least 100 treponemes by darkfield microscopy of wet mounts was in all cases better than 90 %.

Human fibroblasts of TR strain were grown to 25-50 % confluency on 20 x 20 mm cover-slips, kept in plastic petridishes, using F10 medium supplemented with 7 % FCS and 7 % newborn calf serum.

Human fibronectin (FN) was a generous gift from Dr. H.J.G. Brummelhuis of the Central Laboratory of The Netherlands Red Cross Blood Transfusion Services, Amsterdam, the Netherlands. It consisted of 85 % FN, the impurities being mainly albumin and IgG. FN was attached to 20 x 20 mm cover-slips by spreading 50 ul of a solution containing 0.5 mg protein per ml followed by air-drying for at least one hour.

Adherence of the treponemes to monolayers of fibroblasts or to layers of FN was performed by incubating the appropriate cover-slips, kept in 35 mm petridishes with 1.0 ml of T. pallidum suspension during 2 h at 34 °C under a reduced oxygen atmosphere in a dessicator. Non-adherent treponemes were removed by rinsing the cover-slips thrice in a glass beaker filled with phosphate buffered saline (PBS, pH = 7.4). The adherent treponemes were used for immunofluorescence studies. They were used either without further treatment or by analogy to the FTA-ABS reaction, after fixation with acetone (monolayers of fibroblasts and FN layers) or after air-drying (FN layers). Acetone treatment was performed by incubating the rinsed cover-slips during 10 min in dry acetone. The cover-slips were atmospheric air-dried for 10 min. The cover-slips were then rinsed again in PBS and used similarly as untreated cover-slips.

Two sets of sera, one from 5 individual patients with seropositive primary (S 1+) and the second from 5 secondary (S II) stage patients, were used. The criteria for these diagnoses have been published elsewhere (22). Donor sera which were negative for syphilis were used as controls. They were also used in attempts to inhibit the fluorescence by serum. The sera were stored at -70 °C in small aliquots and were used in 5-fold dilutions in PBS.

A C3 deficient human serum and a Clq deficient human serum were obtained from Dr. B.J.M. Zegers (University Children's Hospital, Utrecht). Defective classical as well as alternative pathway of complement activation due to the absence of C3 in a C3 deficient patient has been documented (16). The Clq deficient serum lacked immunochemically detectable Clq, but some functionally active Clq was presumably present as demonstrated by a very low level of lysis of antibody coated sheep erythrocytes. The alternative pathway of complement activation in this serum was not hampered as shown by the AP 50 value which was within normal limits. This indicates the presence of the third complement component. (B.J.M. Zegers, personal communication.)

IgG containing fractions from pooled control sera (IgG(C)) or pooled sera from patients with secondary syphilis (IgG(SII)) were prepared by ion-exchange chromatography on DEAE-Sephadex A-50 columns and eluted with a Tris-HCl buffer at pH = 6.5. The 19 S-peak which was isolated from control (IgM(C)) or secondary syphilis (IgM(SII)) sera by exclusion chromatography on Sephadex G-200 was used as an IgM enriched fraction. These serum fractions were concentrated to the initial volume of the serum from which they were derived. This was achieved using Amicon concentration cells equipped with PM 10 membranes (Amicon, Oosterhout, the Netherlands). The serum fractions (FTA-ABS titers: IgG(SII) fraction: 1:320; IgM(SII) fraction: 1:160) were used in the fluorescence assays in 10-fold dilutions.

A mouse IgM monoclonal antibody (MoAb) to the axial filaments of Nichols pathogenic treponemes (Cc9) was generously supplied by Dr. M. Bailey (University of Birmingham, England).

Conjugates to human C3 and the Fc fragments of human IgG and IgM were obtained from Nordic (Tilburg, The Netherlands). They consisted of the IgG fraction from antisera raised in goats and labeled with fluorescein isothiocyanate (FITC). The conjugate against mouse immunoglobulins was the IgG fraction from a rabbit antiserum against mouse heavy chains and labeled to FITC.(Becton and Dickinson, Mountain View, California, USA) The conjugates were used in a 50-fold working dilution. None of the conjugates reacted directly with the adherent treponemes. No cross-reactivity was observed

between the IgG and IgM conjugates as demonstrated by the negative reactions of the IgG conjugate on adherent treponemes incubated with the IgM(SII) fraction and vice versa.

The immunofluorescence studies were performed on adherent treponemes on the cover-slips which were placed in petridishes. The cover-slips were overlaid with the appropriate dilutions of the sera, serum fractions or monoclonal antibody and incubated for 30 min. They were then rinsed with PBS, and subsequently incubated for 30 min with the appropriate conjugate. They were rinsed again and placed upside down on microscopic slides. The preparations were sealed with nail polish and read immediately. In the experiments performed to inhibit the fluorescence produced by the IgG(SII) and the IgM(SII) fractions by serum, the incubations with the antibody containing serum fractions were either preceded or followed by an incubation by one of the control sera for 30 min.

The slides were examined on a Leitz Orthoplan binocular microscope in alternating darkfield and U.V. illumination. The microscopic equipment and the evaluation of the results read under U.V. illumination were as described previously (22). A minimum of 200 treponemes per slide were examined and the treponemes showing at least a 1+ reaction were considered as positive. The number of positive treponemes were expressed for each slide as a percentage of the total number of treponemes observed.

Results.

Both the fibroblast- and the FN-adhering treponemes on all occasions showed no reaction with the anti-axial filament MoAb Cc9. However, using air-dried or acetone treated preparations all treponemes showed a strong positive reaction with this antibody. The interrupted filamentous pattern of fluorescence was compatible with the structure of the axial filaments twisting around the treponemes. No differences were observed between the fresh and aged treponemes.

The sero-positive primary and the secondary syphilis sera when incubated with the appropriate conjugate, consistently showed no fluorescence for IgG as well as for IgM with the fibroblast- or FN adherent treponemes. This was true for the fresh and the aged treponemes from the twelve isolates tested for IgG, and the six isolates tested for IgM. Only occasionally a very weak fluorescence was observed indicating that some immunoglobulins might have been present. This was especially apparent for IgM antibodies. Air-dried or acetone treated preparations of adherent treponemes showed a strong positive

fluorescence for IgG after incubation with the syphilitic sera and conjugate. However, the treponemes showed only a weak fluorescence after incubation of these preparations with the sera and anti-IgM conjugate.

The results with the immunoglobulin-containing serum fractions are shown in Table 1. When the IgG(SII) fraction was used, a mean of 14.5 % of the fresh treponemes adhering to the fibroblasts showed a positive IgG fluorescence of a 1+ to 2+ intensity. This percentage increased to a mean of 32.1 % when aged treponemes were used. The remaining treponemes were completely negative. When the IgM(SII) fraction was used, a mean of 43.2 % of the fibroblast adhering fresh treponemes showed a bright, strongly positive fluorescence, the remaining treponemes being completely invisible. Using the aged treponemes this increased to a mean of 87.3 %. It can be seen in Table 1, that a greater percentage of the treponemes adhering to the fibroblasts than that adhering to the FN layers showed a positive fluorescence with the IgG(SII) and IgM(SII) fractions. No non-specific binding of immunoglobulins was detected since the controls with IgG(C) and IgM(C), that were run in parallel, were consistently negative.

Table 1.

Percentages of fresh and aged treponemes, which adhere to fibroblast- or fibronectin layers, and which show a positive reaction with IgG or IgM containing serum fractions, isolated from sera of patients with secondary syphilis.

	Fibroblasts			Fibronectin		
	n	fresh	aged	n	fresh	aged
IgG	12	14.5 (8.1)	32.1 (11.7)	12	1.9 (3.0)	13.2 (7.6)
IgM	6	43.2 (15.4)	87.3 (7.9)	6	2.2 (1.6)	52.2 (2.6)

S.D. in parenthesis

n indicates the number of different isolates of treponemes tested.

When the treponemes were pre-treated with acetone or air-dried, they all displayed a bright, strongly positive fluorescence with the IgG(SII) fraction and conjugate. However, with the IgM(SII) fraction only a very weak fluorescence was observed. This was scored as negative.

The discrepancy between the results with whole sera and those with the antibody containing fractions prompted us to investigate whether whole sera interfered with the IgM and IgG fluorescence. Table 2 shows the results of the experiments using four different isolates of treponemes adhering to fibroblasts. The incubation of fresh, fibroblast-adhering treponemes with the control sera before the incubation with the IgG(SII) fraction diminished the percentage of fluorescence-positive treponemes. This procedure had no influence when aged treponemes were used. The IgM fluorescence of the fresh as well as the aged treponemes was almost completely abolished by this treatment. When the incubation of the adherent treponemes with the IgG(SII) or IgM(SII) fractions was followed by an incubation with a control serum, the fluorescence produced by both antibody containing fractions was completely inhibited. Repeated incubations with each of these antibody-containing fractions, followed by the appropriate conjugate resulted in an absence of fluorescence-positive treponemes. This result demonstrated that the access to

Table 2.

Means and standard deviations of percentages of four different isolates of fibroblast-adhering treponemes which show a positive reaction with IgG or IgM containing serum fractions isolated from sera of patients with secondary syphilis.

sequential incubations				fresh	aged
IgG(SII)	aIgG			16.0 (9.9)	34.3 (10.0)
c.serum	IgG(SII)	aIgG		8.8 (5.6)	36.8 (12.5)
IgG(SII)	c.serum	aIgG		0	0
IgG(SII)	c.serum	IgG(SII)	aIgG	0	0
IgM(SII)	aIgM			32.3 (6.0)	97.8 (4.5)
c.serum	IgM(SII)	aIgM		0.8 (1.0)	5.3 (4.5)
IgM(SII)	c.serum	aIgM		0	0
IgM(SII)	c.serum	IgM(SII)	aIgM	0	0

Abbreviations:
aIgG and aIgM indicate the conjugate used.
c.serum indicates control serum.

the treponemal outer membrane was completely obstructed by the components of the control sera.

Heat-inactivation of the control sera diminished, but did not completely abolish their capacity to inhibit the fluorescence of the IgG(SII) or IgM(SII) fractions. This suggested a possible role for complement in the inhibition of fluorescence in the studies using the syphilitic as well as the control sera.

Table 3.

Detection of IgG and IgM class antibodies and the third complement component (C3) on fibroblast-adhering treponemes after various incubations. The means and standard deviations of percentages of three experiments with different isolates are shown.

	Ig		C3	
Sequential Incubations[a]	fresh	aged	fresh	aged
c.serum	n.d.[b]	n.d.	100	100
S II serum	0	0	100	100
Clq def serum	n.d.	n.d.	0	0
IgG(SII)	11.0(8.2)	33.6(6.8)	n.d.	n.d.
IgG(SII)/ c.serum	0	0	100	100
IgG(SII)/ c.serum▲	3.0(2.6)	6.3(3.2)	0	0
IgG(SII)/ C3 def serum	3.7(3.2)	9.7(4.5)	0	0
IgG(SII)/ Clq def serum	0.7(1.2)	1.0(1.0)	24.0(3.5)	52.0(10.1)
IgM(SII)	26.7(5.5)	87.7(5.9)	n.d.	n.d.
IgM(SII)/ c.serum	0	0	100	100
IgM(SII)/ c.serum▲	15.7(7.6)	18.7(9.5)	0	0
IgM(SII)/ C3 def serum	19.0(7.2)	48.7(9.0)	0	0
IgM(SII)/ Clq def serum	1.0(1.7)	0	33.3(10.1)	90.3(8.6)

a. These incubations were followed by one with the appropriate conjugate.

b. applies to IgG as well as IgM class antibodies.

▲ indicates that the serum used was heat inactivated.

n.d. = not done.

As shown in Table 3, the third complement component (C3) was invariably present on all adherent treponemes when they were incubated with syphilitic sera alone or after incubations with IgG(SII) or IgM(SII) followed by one of the control sera. However, the adherent treponemes became also covered by a C3 layer when they were incubated with the control sera alone. After incubation with the Clq deficient serum no C3 was detected on the treponemal surface. However, after the treponemes had been coated with IgG(SII) or IgM(SII), the incubation with the Clq deficient serum resulted in the presence of C3 on a part of the adherent treponemes. The incubation of fresh treponemes with IgG(SII) followed by an incubation with the Clq deficient serum resulted in a proportion of C3 staining treponemes that was almost twice the proportion of IgG staining treponemes after incubation with IgG(SII) alone. Analogous experiments with IgM(SII) resulted in similar proportions of treponemes staining for IgM and C3. In these types of experiments a larger proportion of the aged treponemes than of the fresh treponemes stained for C3. The incubation with C3 deficient serum reduced the proportion of fluorescence-positive treponemes after the pre-incubation with IgG(SII) or IgM(SII) as compared to the serum fractions alone.

Discussion.

The negative results of MoAb Cc 9 with native adherent treponemes show that both fresh and aged treponemes possess an intact outer membrane, which prevents the access of antibodies to the antigens located underneath this membrane. However, the positive results with this MoAb on adherent treponemes after treatment with acetone or air-drying demonstrated that these treatments resulted in a modification of the outer surface of the treponemes which enabled the antibodies to combine with their epitopes on intracellularly located antigens. This demonstrates that untreated, adherent treponemes are a suitable substrate for studying the interactions of antibodies with the outer membrane without the interference of antibodies to intracellularly located antigens.

We have shown that a large part of the fresh treponemes adhering to the fibroblasts or FN did not stain with the IgG(SII) and IgM(SII) fractions. This indicated that the outer membrane of the treponemes acts as an inert barrier for the antibodies. When aged treponemes were used, approximately 10 % of the fibroblast-adhering treponemes were negative for IgM. Aged treponemes adhering to FN-layers showed a mean of 50 % IgM negative treponemes. The percentages of

the aged treponemes that were IgG negative were 68 % for fibroblast-adhering treponemes and 87 % for FN-adhering treponemes. The increase in the percentages of staining treponemes on aging is compatible with the changes in the outer surface of the treponemes. This may concern changes in the outer membrane itself perhaps due to insufficient in vitro metabolic capacity of the treponemes to maintain the inertness of the outer membrane or due to a loss of an extracellular cover of the outer membrane.

Using the IgG(SII) fraction consistently lower percentages of fluorescence-positive treponemes were detected than using the IgM(SII) fraction. This did not change when a four-fold concentrated IgG(SII) fraction was used. We have previously shown that during the S II stage the IgG production reached its maximum (19,22). Moreover, this fraction produced strong positive reactions on acetone-treated or air-dried preparations. The differences between the staining with both serum fractions might be explained by the multivalency of the IgM antibodies, which enable them to bridge larger distances between epitopes than the IgG antibodies. Alternatively, the differences might be explained by the assumption that the production of IgG antibodies to the outer membrane components is less extensive than the production of IgM antibodies. Moreover, it is not known whether the lower numbers of IgG positive treponemes are due to the inaccessability of the relevant antigens on the membrane or are due to the absence of IgG antibodies to some outer membrane components or epitopes. A suppressed IgG response to the T-cell dependent, unrelated sheep red blood cell antigen during a syphilitic infection in rabbits has been reported (5,6). Further studies should differentiate between these possibilities.

The differences between fibroblast and FN layers with respect to the percentages of positively staining, adherent treponemes might be related to the larger diversity of ligands available on the fibroblasts than on FN. As demonstrated by Fitzgerald et al (10) treponemes adhere not only to FN, but also to collagen type I and IV, laminin and hyaluronic acid. Exposed sites on the outer membrane of the treponemes may be responsible for the adherence to these substances and at the same time for the larger percentage of positively staining treponemes.

The negative results for IgG and IgM fluorescence with the syphilitic sera could be reproduced by using sequential incubations with the IgG(SII) or IgM(SII) fractions and control sera. This was invariably accompanied by the presence of a layer of C3 around all the treponemes. Interestingly, the incubation with the control sera alone also resulted in a C3 layer around all

the treponemes present. Therefore, the inability to detect IgG- or IgM class antibodies on the treponemal surface might be due to the binding of C3 to the outer membrane, thereby inhibiting the access of the antibodies or due to the blocking of antibodies, that bind to the treponemes and that are subsequently covered by C3. A C3 layer was absent when the treponemes were incubated with a Clq deficient serum. This excludes a complement deposition via the alternative pathway as well as a spontaneous binding of C3 to the treponemal surface and favours the classical pathway of complement activation as a cause of the C3 deposition. The pre-incubations of the treponemes with IgM or IgG antibodies, followed by the incubation with the Clq deficient serum resulted in a C3 layer around a part of the treponemes. The reason for this is unknown. It might reflect the low level of functionally active Clq in this serum. Alternatively, the C3 deposition might be caused by a complement activation via the alternative pathway. The use of either heat-inactivated sera or a C3 deficient serum in the second incubation after the treponemes had been coated with IgG(SII) or IgM(SII) did not result in a C3 layer. This restored only partially the possibility to detect the antibodies on the treponemal surface. However, at present it is unknown whether the lower numbers of positively staining treponemes, as compared to the numbers obtained with the antibody containing serum fractions alone, were due to a covering of the antibodies by serum proteins other than C3 or to a loss of antibodies during the second incubations. Repeated incubations with antibody fractions and appropriate conjugate to demonstrate a possible inaccessability of the treponemal outer membrane were inconclusive.

Assuming that the treponemes do not posess an intrinsic mechanism of complement activation, the C3 deposition from the control sera suggest that antibodies may be present on the treponemal surface. These antibodies must have been derived from the rabbits from which the treponemes had been isolated. However, our attempts to detect these antibodies by means of fluorescence failed. An explanation might be that they are burried in an extracellular layer and that other techniques are needed for their detection. Indeed, several authors have shown that C3, IgG and IgM were among the host serum proteins present on the treponemal surface (2,4). However, it remains puzzling to us how these antibodies remained undetected by our fluorescence technique while at the same time, they are able to activate complement.

In conclusion, our results demonstrate that a large part of the fresh adherent treponemes are unable to react with the antibodies isolated from the sera of syphilitic sera. The most likely explanation seems to be that an

extra-cellular layer inhibits the access of these antibodies to the treponemal outer membrane. The deposition of C3 from the control sera suggests that rabbit antibodies may constitute a part of this extra-cellular layer. The occupation of epitopes by these rabbit antibodies might form an additional explanation for the inability of the adherent treponemes to react with the antibody fractions from the syphilitic sera. However, the failure to demonstrate these rabbit antibodies by immunofluorescence suggests that the technique used is not sensitive enough or that they are burried in this extra-cellular layer. Further investigations will require purified treponemes that are free of host components in order to elucidate the manner in which the various host components contribute in the formation of an extra-cellular layer, thus allowing the treponemes to escape the action of antibodies and complement.

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CHAPTER 9.

SUMMARY AND GENERAL DISCUSSION

The objective of the study described in this thesis was to investigate the possible mechanisms which enable the pathogenic treponemes to escape from the host defences. The literature findings available at the start of the current investigations suggested a suppression of the immune system, particularly the T cell-mediated response during syphilis. Since a normal and efficient antibody production requires the cooperation between the T and B cells, the development of the IgG response was used as an indicator to investigate such a suppression during syphilis in man.

In Chapter 4 it was shown that the production of aspecific IgG class anti-treponemal antibodies as demonstrated by the FTA-test occurred very rapidly. The anti-treponemal antibodies belonging to the three quantitatively most important IgG subclasses, were already detectable during the earliest diagnostic stage, the S 1^{-} stage. This indicates that the capacity of the immune system to initiate and, in later stages, to maintain the production of IgG antibodies is not affected by the treponemal infection and therefore does not support the hypothesis of immunosuppression. However, the specific anti-treponemal antibodies as delineated by the FTA-ABS test showed a sequential development of the IgG subclasses. They were detected first during the sero-changing stage and were then almost completely restricted to the IgG1 and IgG3 subclasses. IgG2 subclass antibodies were present first during the sero-positive primary stage. The preference for the production of the IgG class anti-treponemal antibodies during early syphilis in the basic part of heterogeneous IgG (1) was confirmed by the titration of three electrophoretically different IgG fractions for the presence of IgG1 subclass antibodies. During primary syphilis these subclass antibodies were almost completely confined to the most basic fraction. Only during the secondary stage they were present in high titers in each of the three fractions. Upon immunoblotting of solubilized treponemes, other investigators (2,3,4) showed that the sera from patients with primary syphilis contained IgG and IgM antibodies against a limited number of the antigens only, while the sera of patients with secondary syphilis contained IgG and IgM class antibodies against all polypeptide bands. The slow development of the IgG response

together with the late involvement of the treponemal antigens in the production of IgG suggests that the sensitization against these antigens occurs late in the course of the disease. Therefore, it seems likely that until the secondary stage of the disease, a well functioning immune system is not confronted with the majority of the treponemal antigens. This may be either due to their location inside the treponemes or sequestration by a protective layer or both.

The histopathological studies on infected rabbit testes, described in Chapter 5, were performed to gain insight into the in vivo location of T. pallidum and the nature of the deposited mucinous substances. An impressive observation in these studies was the destruction of the normal architecture of the testes with the concomitant formation of large optically empty areas. The presence of mucopolysaccharides in these areas was demonstrated by the Alcian Blue and HIDAB staining methods. The treponemes which were visualized by silver impregnation methods showed a preferential location in the areas rich in mucopolysaccharides. The Warthin staining followed by Alcian Blue staining revealed the close association of the treponemes with pre-existing tissue fibrils and the cells contained within these fibrils. This close association was confirmed by electronmicroscopy. These findings emphasize the significance of the in vitro adherence of the treponemes to cultured tissue cells. The nature of the mucopolysaccharides that are produced during the treponemal infection are of interest in view of their possible involvement in the formation of an extra-cellular cover around the treponemes (5,6). It is still a matter of doubt whether these mucopolysaccharides are produced by the treponemes themselves or are synthesized by the host in response to the presence of the treponemes. The differential staining of sections from the infected testes using the HIDAB method showed the presence of large amounts of sulphated mucopolysaccharides. Previous studies by others suggested that these were chondroitin sulphates (7). They were contained within fibrils between which there were empty spaces. The blue staining by the HIDAB method which indicates the presence of hyaluronic acid, was observed only in approximately half of the infected testes.

In Chapter 6, it was shown that the extraction of the infected testes yielded suspensions that contained, in addition to T.pallidum, variable amounts of mucopolysaccharides. Further analysis of these mucopolysaccharides showed that only hyaluronic acid was present, chondroitin sulphates and other types of mucopolysaccharides were not detected. Assuming that the deposition of mucopolysaccharides was responsible for the oedema in the infected testes

leading to an increase in their weight, a discrepancy was noted between the amount of mucopolysaccharides in the testicular extracts and the wet weight of the testes after infections of 7 and 12 days duration. This suggested that the formation of hyaluronic acid was preceded by the formation of non-extractable mucopolysaccharides and that the sulphated mucopolysaccharides were firmly bound to the ground substance. The fibrillar structure observed after HIDAB staining supports this suggestion. The easy extractability of hyaluronic acid suggested a loose binding of this compound. Since the sulphated mucopolysaccarides of uninfected tissue are bound predominantly to serine residues of the core protein via their linkage units (8) a further analysis of their way of binding to the core protein would be helpful in determining the origin of these mucopolysaccharides. Since in vitro studies have shown that the adherence of treponemes to tissue culture cells resulted in the deposition of mucopolysaccharides (6), it seems feasible, in view of the close association in vivo between the fibroblasts and the treponemes that the latter could stimulate the fibroblasts to produce one or both types of mucopolysaccharides. A further speculation is that the treponemes are able to convert chondroitin sulphate into hyaluronic acid.

The study on the in vitro adherence of the treponemes to cultured human fibroblasts are described in Chapter 7 and the addendum to this Chapter. First, the generally accepted opinion that only viable treponemes adhere to tissue culture cells (10) was confirmed using fresh treponemes. Treponemes which had been killed on the day of their isolation and which had been used either the same day or the next day failed to adhere. However, viable treponemes that had been stored for 24 h (aged treponemes) and were then killed had retained the capacity to adhere, albeit in lower numbers than those from their viable parent suspensions. Thus, it seems that during the in vitro storage of the treponemes, changes occurred in their outer surface, which were the result of an active process that depended on their viability. The adherence on the first day was mediated by an active process, while on the second day it could have occurred (in part) passively. Furthermore, the aged treponemes adhered in almost twice as large numbers as did the fresh treponemes. The sera from the patients with syphilis partially inhibited the adherence of the fresh as well as the aged treponemes. This capacity was first demonstrable in the sera of patients at the end of the primary stage. Sera from patients of earlier stages did not exert any adherence inhibition. The sera of patients with secondary syphilis showed the greatest adherence inhibition capacity. This was also demonstrable in the sera of patients with

late latent syphilis. These results showed that from the end of the primary stage onwards the sera of syphilitic patients had a greater adherence inhibition capacity than that of the control sera. Other investigators have attributed this to anti-treponemal antibodies (9,10,11). However, in view of the known interaction of several serum proteins with T. pallidum (12,13) and of the results described in Chapter 8, it was doubtful whether antibodies constituted the (only) adherence inhibition factor of the syphilitic sera. The role of fibronectin as the major ligand of the extra-cellular matrix of the cells in the adherence of the treponemes prompted us to investigate whether fibronectin in the sera of syphilitic patients participated in the adherence inhibition of the treponemes. To this end, the sera were fractionated by Sephadex G-200 gel filtration. When fresh treponemes were used, the adherence inhibition factors appeared to be located in the serum fraction containing the macromolecular species. This was compatible with a role of both fibronectin as well as IgM in the adherence inhibition. When this serum fraction was absorbed with gelatin-sepharose, its adherence inhibition capacity was lost, but its relative IgM content was not affected. This showed that fibronectin was the major serum component that inhibited the adherence of the fresh treponemes. The fraction 1 inhibited the adherence of aged treponemes to a greater extent than that of fresh treponemes, but the absorbtion with gelatin-sepharose did not restore the adherence of the aged treponemes to control levels. Moreover, the fraction 2 from the sera of patients with secondary syphilis also contained a weak adherence inhibition capacity towards aged treponemes in contrast to the comparable fraction from the sera of patients with sero-positive primary syphilis. Attempts to remove the adherence inhibition factors from the sera of syphilis patients by pre-absorption with treponemes appeared to be succesfull only when the absorbed sera were used with fresh treponemes. When aged treponemes were used, no loss in the adherence inhibition capacity of the absorbed sera was observed. These phenomena were compatible with a role of antibodies only when aged treponemes were used.

Whether there is any relationship between the adherence of treponemes and their ability to provoke disease is unknown at present. A role of adherence in the establishment of the infection at local sites can easily be envisaged. A second role might be derived from the succesful in vitro propagation of the treponemes using a co-cultivation tissue culture technique (14). This opens the possibility that in one way or the other, the adherence to the cells might be necessary for the completion of the reproduction cycle of the treponemes. The interference with the adherence in vivo might prevent the location of the

treponemes at distant sites and thus limit their number. Since the freshly isolated treponemes presumably bear the best resemblance to the treponemes occurring in vivo, the in vitro studies show that fibronectin, but not antibody, is the most likely component of serum that is capable to interfere with the in vivo adherence of the treponemes. A role of antibodies in this respect seems unlikely since their activity was only exerted on the aged treponemes. One could speculate further that the interaction of the treponemes with fibronectin prevents the in vivo adherence to tissue cells. This could possibly lead to the exposure of the antigens on the outer membrane and could, in turn, lead to the induction and the production of antibodies. This sequence of events is supported by the observation that the stage of the disease during which the adherence inhibition by serum is detected first, precedes the stage during which a full complement of anti-treponemal IgG1 subclass antibodies is present. Further investigations will have to prove a causative relationship between these two phenomena.

In Chapter 8 the question whether antibodies had access to the treponemal outer membrane was investigated by means of an indirect immunofluorescent technique using the treponemes that had attached spontaneously to layers of fibroblasts or fibronectin. The absence of fluorescence using a monoclonal antibody, directed against the axial filaments of the pathogenic Nichols treponemes assured that these adherent treponemes possessed an intact outer membrane. The IgG or IgM containing fractions isolated from the sera of patients with secondary syphilis, showed no fluorescence in the majority of the adherent fresh treponemes. This demonstrated the inertness of the outer membrane in the binding of antibodies. When aged treponemes were used the percentage of IgM reactive treponemes increased to almost 100 %, the percentage of IgG reactive treponemes increased too, but to a lesser extent. This shows that changes occurred in the outer membrane during in vitro aging of treponemes. After the incubation of the adherent treponemes with the sera of patients with sero-positive primary or secondary syphilis no binding of antibodies by either the fresh or the aged treponemes was observed. Similar results were obtained when the adherent treponemes were incubated with either IgG or IgM antibodies isolated from pooled secondary syphilitic sera followed by an incubation with control sera. This was invariably accompanied by the deposition of a third complement component (C3) layer around the treponemes. When heat-inactivated control sera, a C3 deficient serum or a C1q deficient serum were used, no deposition of C3 occurred, but an incomplete inhibition of fluorescence was observed. Interestingly, the incubation of the fresh or aged

adherent treponemes with the control sera alone also resulted in the deposition of a C3 layer around the treponemes. However, after the incubation with the Clq deficient serum such a C3 layer could not be demonstrated. This excludes the activation of complement via the alternative pathway as well as the spontaneous absorption of the native complement as the possible causes of the C3 deposition. Assuming that the treponemes do not possess an intrinsic mechanism for complement activation, the most probable cause of the C3 deposition is the activation of complement via the classical pathway. This indicates that the treponemes presumably carry antibodies of rabbit origin on their surface, which are not lost during in vitro aging. However, immunofluorescent studies using the appropriate conjugates failed to detect these rabbit antibodies. It is also possible that this technique may not be sensitive enough since several rabbit serum components, including IgM, IgG and C3 were detected using various other techniques (13,15).

A common observation in Chapters 7 and 8 was the occurrence of changes in the outer surface of the treponemes upon in vitro aging. This was demonstrated by the increase in the numbers of treponemes that adhered per fibroblast, the adherence inhibition by antibodies and the access especially of IgM class antibodies to the outer membrane of the aged treponemes as compared to the fresh treponemes. It was shown that these changes occurred only on viable treponemes. The occurrence of these changes can be explained as follows. First, it may be a phenomenon that is observed only in vitro and is due to the insufficient metabolic activity to maintain the inertness of the outer membrane in treponemes kept in vitro. In this case only the experimental results obtained using fresh treponemes are relevant to the in vivo situation. A second explanation is that the binding of host components (e.g. hyaluronic acid and/or serum proteins) to the components of the outer membrane result in its inertness. The loss of inertness in vitro could be due to a simple loss of the bound host components. However, since killed fresh treponemes after storage do not acquire the capacity to adhere to fibroblasts this seems unlikely. Thirdly, the maintenance of the inertness of the outer membrane could be regulated via a dynamic process whereby the outer membrane components, together with any bound host components are shed and replaced. In vitro release of small treponemal polypeptides by viable treponemes has been previously shown (16). In their study on the mechanism of the TPI reaction, Müller et al (17) demonstrated that the integrity of the treponemal outer membrane was affected after the addition of IgG class antibodies and complement, but that the treponemes were capable of repairing the outer

membrane. Such a release of outer membrane components in vivo could explain the initial IgG and IgM production against a limited number of treponemal antigens and also the occurrence of antibodies on an otherwise protected outer surface. Furthermore, the release of outer membrane components together with antibodies would result in circulating immune complexes. In Chapter 8 evidence for the presence of antibodies of rabbit origin on the treponemal surface has been presented. Such complexes might interfere with the production of IgG antibodies. In this connection the production of IgG class antibodies against the outer membrane components could be suppressed. This is not necessarily contradictory to the results of previous investigations (Chapter 4), which failed to provide evidence for a suppression of the IgG response. It must be noted that the latter were performed using a full range of treponemal antigens. Evidence for the possibility that the antibody production against the components of the outer membrane within the IgG class was diminished as compared to the production of IgM class antibodies was presented in Chapter 8. When aged treponemes were used, the IgM antibody fraction isolated from the sera of patients with secondary syphilis stained almost all treponemes present, while the comparable IgG fraction stained a considerably lower number. Further evidence stems from the restricted local IgG production in comparison to the local IgM production in infected rabbit testes (18). Furthermore, the dynamic character of the treponemal outer membrane could decide on localization or dissemination of the treponemes. These phenomena could be dependent on a covering or an exposure of the relevant treponemal binding sites. A different function of the antibodies on the treponemal surface could be that the combination of these antibodies with certain key antigens can inactivate the reproductive cycle of the treponemes. In patients with late latent syphilis, a waxing and waning of the titers of anti-treponemal antibody titers has been noted (19). This could reflect the dynamic equilibrium between the multiplication of the residual treponemes during phases of low antibody concentrations and the inactivation of the treponemes as a result of the increasing antibody titer during the following period. In the passive immunization experiments, the delay in the development of lesions in passively protected rabbits as compared to non-protected control animals is usually attributed to the effective eradication of the treponemes by antibodies and complement. However, the delay in the development of lesions can be equally explained by the assumption that the treponemes are temporarily inactivated by the passively administered antibodies. In fact, once the administration of the immune serum is stopped, the protected rabbits developed

the lesions after a similar lapse of time as that observed in the control animals. After one year all the immune serum protected rabbits suffered from an asymptomatic infection (20). The conclusion of Perine et al (21) that "....the effect of the serum was primarily that of preventing an increase in numbers of organisms: at least its effect was not one of killing all or even the majority of the organisms" can easily be understood from the above mentioned assumption. Unfortunately, no in vitro cultivation method of the treponemes is as yet available to test this hypothesis. However, if confirmed, a serious risk of future vaccinations would be the abortion of the symptomatic stages of the disease. In that case the disease would follow an asymptomatic course. This would diminish the possibility of early diagnosis and effective treatment and increase the risk for the development of the late complications of the tertiary stage.

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SAMENVATTING.

Het doel van de studie beschreven in dit proefschrift was na te gaan op welke wijze pathogene treponemata in staat zijn te ontsnappen aan de afweer van de gastheer. Het proefschrift is samengesteld uit een tweetal literatuur overzichten en vijf experimentele studies.

In Hoofdstuk 1 is een overzicht gegeven over de aard en het voorkomen van de verschillende treponematosen en van hun verwekkers. Verder zijn de biochemische en biologische karakteristieken van de treponemata besproken. De gegevens zijn voor het belangrijkste deel ontleend aan studies met Treponema pallidum, de verwekker van syphilis. De onmogelijkheid tot dusver om de treponemata in vitro in voldoende grote aantallen nodig voor verder onderzoek te kweken vormt een grote belemmering zowel bij de interpretatie van de in de literatuur voorhanden zijnde onderzoeksgegevens als bij verdere studies.

In Hoofdstuk 2 wordt een overzicht gegeven van het ziektebeeld syphilis en de bijbehorende histopathologische en immunologische bevindingen, zowel bij de mens als bij proefdieren. Deze gegevens geven geen steun aan de, bij de aanvang van deze studie veelvuldig in de literatuur aangetroffen hypothese, dat ten gevolge van de treponemale infectie bij syphilis een onderdrukking van het immuunapparaat optreedt. Deze immunosuppressie zou dan verantwoordelijk zijn voor de overleving van de treponemata binnen de gastheer. Hiermee zou dan het optreden van de late verschijnselen kunnen worden verklaard.

In Hoofdstuk 3 is een overzicht van de literatuurbevindingen gegeven, die hebben geleid tot het, eveneens in dit hoofdstuk geformuleerde, doel van de studie.

Hoofdstuk 4 beschrijft een onderzoek naar de ontwikkeling van anti-treponemale antilichamen in de vier immuunglobuline G subklassen met behulp van de FTA- en de FTA-ABS testen bij patienten met syphilis. Bij gebruik van de FTA-test, waarmee zowel specifieke als aspecifieke antilichamen worden opgespoord, worden reeds in het vroegst diagnostiseerbare stadium van syphilis anti-treponemale antilichamen gevonden in de drie in kwantitatief opzicht belangrijkste IgG subklassen. Bij gebruik van de FTA-ABS test blijken de antilichamen het eerst gevonden te worden in de IgG1 en IgG3 subklassen. De titratie van anti-treponemale IgG1 subklasse antilichamen in -in lading verschillende- IgG fracties bevestigde de suggestie dat deze antilichamen gedurende de primaire stadia hoofdzakelijk gevonden worden in de meest basische delen van het heterogene IgG en pas tijdens het secundaire stadium

van de ziekte in de volledige IgG heterogeniteit aanwezig te zijn. Deze bevindingen tonen aan dat uit de IgG response geen suppressie van het immuunapparaat blijkt, doch dat de vorming van IgG antilichamen langzaam verloopt.

Hoofdstuk 5 is een studie naar de localisatie van Treponema pallidum in geinfecteerde konijnentestes en de aard van de tijdens de infectie gevormde zure mucopolysacchariden. Licht- en electronen microscopische studie tonen aan dat in dit weefsel de treponemata in een nauwe associatie met gastheerweefsel worden aangetroffen. Dit ondersteunt de waarde van de in vitro bevindingen van de adherentie van de treponemata aan weefselkweekcellen. Langs histochemische weg kon worden aangetoond, dat tijdens de infectie zure mucopolysacchariden worden gevormd. Deze konden worden gedifferentieerd in het gecarboxyleerde mucopolysaccharide hyaluronzuur en gesulfateerde mucopolysacchariden, vermoedelijk chondroitinesulfaten.

In Hoofdstuk 6 zijn de experimenten beschreven waarmee werd aangetoond dat de suspensies welke door extractie van T. pallidum uit van te voren geinfecteerde konijnentestes werden verkregen mucopolysacchariden bevatten. Hyaluronzuur bleek het enige hierin aan te tonen mucopolysaccharide te zijn. Na infecties van 12 dagen werd een grotere hoeveelheid hyaluronzuur geisoleerd dan na infecties van 7 dagen. Deze milde extracties met het overlevingsmedium voor T. pallidum doen vermoeden dat het hyaluronzuur slechts los gebonden voorkomt in dit geinfecteerde weefsel, terwijl chondroitine sulfaat op een andere wijze is gebonden. Tezamen met de microscopische beelden betreffende het voorkomen van mucopolysacchariden suggereert dit dat de gesulfateerde mucopolysacchariden deel uit maken van de bindweefselgrondsubstantie. Dit leidt tot de suggestie dat de interactie van treponemata met de gastheercellen zou kunnen leiden tot een stimulatie van de mucopolysacchariden productie door de fibroblasten.

Hoofdstuk 7 beschrijft de studie naar de in vitro adherentie van treponemata aan weefselkweekcellen en de ontwikkeling van factoren in het serum van syphilis patienten, die deze adherentie beinvloeden. Treponema pallidum bleek te hechten aan gekweekte humane fibroblasten, hetgeen in overeenstemming is met de resultaten van andere onderzoekers. Hierbij bleek dat de in vitro verouderde treponemata in bijna twee maal zo grote aantallen aanhechtten als de vers geoogste treponemata. Serum van syphilis patienten met seropositieve primaire syphilis en van patienten met latere stadia waren in staat tot een partiele inhibitie van de adherentie, waarbij het effect op verouderde treponemata groter was dan op verse treponemata. Sera van patienten

met secundaire syphilis vertoonden een maximale capaciteit tot adherentie-inhibitie. Sommige vers geoogste treponema-suspensies waren ongevoelig voor de invloed van de adherentie inhibitie factoren in de syphilitische sera, hetgeen werd opgeheven door in vitro veroudering. Er kon worden vastgesteld dat dit zijn oorzaak vond in de treponemata en niet in de gebruikte sera.

In het Addendum bij Hoofdstuk 7 is getracht vast te stellen of inderdaad alleen levende treponemata tot aanhechting in staat zijn en is onderzocht welke factoren in het serum van syphilis patienten verantwoordelijk zijn voor de door deze sera veroorzaakte inhibitie van de adherentie. Wat de vers geoogste treponemata betreft werd gevonden dat zij in leven dienen te zijn om adherentie te vertonen. De op de dag van isolatie gedode treponemata, hetzij gebruikt op dezelfde dag, hetzij de volgende dag, hadden hun vermogen tot aanhechting verloren. Echter, treponemata die werden gedood de dag na isolatie hadden dit vermogen gedeeltelijk behouden. Dit duidt erop dat de adherentie op de eerste dag afhankelijk is van het in leven zijn van de treponemata en plaats vindt in een actief proces, terwijl dit na veroudering een passief proces kan zijn. Door gebruik te maken van verschillende scheidingstechnieken voor serum eiwitten werd gevonden dat de adherentie inhibitie factoren zijn gelocaliseerd in een hoog moleculaire fractie. Verder werd het aannemelijk gemaakt dat de adherentie inhibitie door fibronectine wordt veroorzaakt en dat immuunglobulinen hierbij slechts in geringe mate een rol spelen.

In Hoofdstuk 8 is de toegankelijkheid van de outer membrane van Treponema pallidum voor anti-treponemale antilichamen bestudeerd, waarbij gebruik gemaakt werd van een indirecte immunofluorescentie techniek. De spontane adherentie van de treponemata aan fibroblasten- en aan fibronectine-lagen maakte het mogelijk de treponemata te fixeren zonder gebruik te maken van mogelijk schadelijke fixatiemiddelen. De integriteit van de outer membrane bleek uit het niet reageren van een monoclonaal antilichaam gericht tegen de axiale filamenten van de treponemata. Van de vers geoogste, aangehechte treponemata bleek slechts een minderheid toegankelijk te zijn voor de IgG en IgM antilichamen uit desbetreffende fracties, geisoleerd uit de sera van patienten met secundaire syphilis. Bij gebruik van in vitro verouderde treponemata werd een toename in deze percentages gevonden. Werden de sera van patienten met seropositieve primaire en secundaire syphilis gebruikt, dan werd geen binding van IgG of IgM antilichamen aangetoond. Echter, de incubaties met deze sera, evenals die met controle sera gingen gepaard met een depositie van de derde complement factor (C3) op het oppervlak van de treponemata. De afwezigheid van C3 na een incubatie met een Clq deficient serum sluit zowel

een spontane absorbtie van C3 als een activatie van de complement cascade via de alternatieve weg uit. De C3 depositie is waarschijnlijk het gevolg van complement activatie via de klassieke weg. Dit zou erop kunnen wijzen dat antilichamen van konijnen oorsprong op het treponema oppervlak aanwezig zijn. Deze zouden deel uit kunnen maken van een beschermende extra-cellulaire laag.

In Hoofdstuk 9 is een samenvatting van de voorgaande hoofdstukken en een meer of minder speculatieve interpretatie van de verschillende resultaten gegeven. Een belangrijke, langs meerdere wegen vastgestelde bevinding is dat vers geoogste treponemata, althans in vitro, niet toegankelijk zijn voor antilichamen. Een verklaring hiervoor kan zijn dat de ruimtelijke orientatie van de outer membrane eiwitten zodanig is dat de immunogene delen hiervan niet worden bloot gesteld. Een tweede verklaring kan worden gevonden in de aanwezigheid van een extra-cellulaire laag, welke de outer membrane afschermt. Mogelijke bestanddelen van deze laag zijn blijkens dit onderzoek mucopolysacchariden, met name hyaluronzuur en (serum) eiwitten van de gastheer.

DANKWOORD

Aan allen, die aan het tot stand komen van dit proefschrift hebben mee gewerkt wil ik mijn hartelijke dank betuigen.

De onderzoekslijn waarbinnen het in dit proefschrift beschreven onderzoek valt werd gestart door Prof. Dr. C.H. Beek. Professor Beek, U bent er in geslaagd Uw enthousiasme voor het treponema onderzoek op mij over te dragen.

Prof. Dr. E. Stolz. Beste Ernst, ik ben je dankbaar voor enerzijds de grote vrijheid en tegelijkertijd de steun bij het voortzetten van dit onderzoek, anderzijds voor de door jou op mij uitgeoefende druk de resultaten ervan in een proefschrift vast te leggen.

Prof. Dr. H.G. van Eijk. Beste Henk, hartelijk dank voor het feit dat je mijn promotor hebt willen zijn. De ontspannen manier van overleg en de grote snelheid waarmee je de verschillende versies van commentaar hebt voorzien, hebben een stimulerende invloed gehad.

Hooggeleerde Michel, Hooggeleerde van der Heul. U beiden ben ik erkentelijk voor het kritisch doornemen van het manuscript.

Mijn dank gaat uit naar de (oud-)medewerkers van het Research Laboratorium Dermato-Venereologie, die allen met inzet hun steentje aan het treponema-onderzoek hebben bijgedragen. In dit verband dienen Marijke Boer, Emma van Reede, Grietje van Dijk en Hans Koehorst te worden vermeld. Meneer Onvlee verdient als free-lance medewerker een aparte eervolle vermelding voor de wijze waarop hij ons heeft laten profiteren van zijn jarenlange ervaring in het omgaan met treponemata. Bob Tank ben ik veel dank verschuldigd voor het aanbrengen van veelvuldige correcties in mijn gebruik van zijn moedertaal.

Het (oud-)verplegend personeel van de "prikunit" van de venereologische polikliniek ben ik erkentelijk voor de zorg waarmee, zij het soms na enige aandrang, zij de bloedmonsters van de patienten hebben verzorgd.

De medewerkers van het Rijks Instituut voor Volksgezondheid en Millieuhygiene te Bilthoven wil ik bedanken voor de prettige wijze waarmee met hen kon worden samengewerkt. Ron van Eijk, Jan van Embden, Gert Aelbers, Jan Compeer, Rien van Niekerk en Cleem Achterberg waren steeds bereid de gevraagde hulp te bieden. Ik hoop deze samenwerking in de toekomst te kunnen voortzetten.

Ook de medewerkers van de Afdeling Klinische Pathologie ben ik veel dank verschuldigd. Thea Zwitser, Johan van Lier en Piet van der Heul hebben zich, ieder op hun terrein, zeer gewaardeerde inspanningen getroost. Het overleg

over het onderzoek met Vojislav Vuzevski en Fibo ten Kate verliep steeds zeer vlot. De vele uren, vaak buiten de normale werktijden, die met Fibo achter het microscoop werden doorgebracht waren voor mij zeer leerzaam.

Paula Delfos en Johan van der Stek hebben op uitstekende wijze de fotografie verzorgd.

Tot slot is een woord van dank op zijn plaats aan de vele patienten, die desgevraagd bereid waren belangeloos bloed voor onderzoek af te staan.

CURRICULUM VITAE

De schrijver van dit proefschrift werd op 21 augustus 1938 geboren te Assen. In 1957 werd het einddiploma HBS-B aldaar behaald. In 1963 werd begonnen met de studie chemie aan de Rijks Universteit te Utrecht, waar in 1966 het kandidaatsexamen, studierichting g, werd afgelegd. Het doctoraal examen, met als hoofdvak biochemie (Prof. Dr. L.L.M. van Deenen) en bijvak immunochemie (Prof. Dr. R.E. Ballieux), werd in januari 1971 behaald. Sedert juni 1971 is schrijver werkzaam op het Research Laboratorium van het Instituut Dermato-Venereologie van de Medische Faculteit aan de Erasmus Universiteit te Rotterdam, thans als universitair docent. Op genoemd laboratorium werd dit proefschrift bewerkt.